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RISK FACTORS AND SPREAD OF *CYSTOISOSPORA SUIS* AND *CRYPTOSPORIDIUM SUIS* IN FARMS OF ODESA REGION

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Summary. This study aimed to determine the prevalence of *Cystoisospora suis* and *Cryptosporidium suis* oocysts in fecal samples from suckling piglets in farms in the southern and northern districts of Odesa Region, to assess the consistency of feces and oocysts, as well as risk factors associated with the hydrothermal regime of the area. Cystoisospores were detected by the McMaster method, and cryptosporidia by the Kester and Romanowski-Giemsa method, followed by microscopy at 630× magnification. In the farms of Bolhrad District, the total infection with cystoisospores and cryptosporidia was 34.5%, while in the farms of Podil District it was 42.2%. The isolation of *Cystoisospora suis* oocysts was high in both districts, ranging from 27.2 ± 0.4 to 32.1 ± 0.5 oocysts per 10 microscope fields of view. In comparison, the main intensity of *Cryptosporidium suis* ranged from 13.6 ± 0.2 to 19.8 ± 0.6 oocysts per 10 microscope fields of view. In the farms of the southern part of Odesa Region, which was characterized by a very severe drought in 2023, *Cystoisospora suis* was most frequently detected in sucking pigs (27.6%). Oocysts of *Cystoisospora suis* were found in 82.7% of liquid feces and 17.3% of solid feces. In the farms of the northern part of Odesa Region with sufficient humidity, *Cryptosporidium suis* was most frequently detected in suckling pigs (25.4%). In the liquid feces of 27.7% of piglets *Cryptosporidium suis* oocysts were detected with the main intensity from 7.3 ± 0.2 to 9.1 ± 0.1 oocysts per 10 microscope fields of view, while in the formed feces of 72.3% of piglets, the main intensity of the infection was from 13.2 ± 0.1 to 16.1 ± 0.1 oocysts per 10 microscope fields of view

Keywords: sucking piglets, cystoisosporiasis, cryptosporidiosis, prevalence, main intensity

Introduction. Internal parasites are prevalent in pigs worldwide and can cause clinical disease or subclinical infections with negative consequences such as poor weight gain and reduced well-being, affecting productivity (Pettersson et al., 2021).

Neonatal cystoisosporiasis is a widespread and important disease of suckling piglets in modern farming systems caused by *Cystoisospora suis*. The impact of endoparasites depends on the parasitic load and individual animal resistance, which can be influenced by environmental and nutritional factors (Gong et al., 2021; Bohach, 2024).

Cystoisospora suis develops entirely in a single host. After entering the environmentally stable oocyst stages, a complex development begins, during which the infective stages are released and infect the small intestinal epithelial cells for asexual reproduction in the intracellular vacuole. After this phase of rapid reproduction (merogony), the parasites differentiate into sexual stages, after which cell fusion occurs and a zygote is formed. The zygote forms the wall of the oocyst and is released in an immature state. In the external environment, maturation occurs and the life cycle is completed. The rapid development of *Cystoisospora suis* (3–5 days) ensures rapid spread within and between groups of newborn piglets (Joachim and Shrestha, 2019).

Cryptosporidiosis is considered the most important zoonotic disease caused by globally prevalent parasitic protozoa called *Cryptosporidium* spp. Cryptosporidiosis is becoming a serious public health and veterinary problem as it affects humans and various animal species. The oocyst stage of *Cryptosporidium* spp. can remain infectious and is resistant to different environmental influences (Pumipuntu and Piratae, 2018).

Among the animals susceptible to *Cryptosporidium*, pigs are considered one of the main host reservoirs (Qi et al., 2020).

Although diarrhea is the most common manifestation of these diseases, subclinical cases are often observed. In both situations, the parasite damages the intestinal mucosa, leading to intestinal dysfunction and decreased productive parameters such as average daily gain and feed conversion (Helmy and Hafez, 2022).

Contaminated farrowing cages are the main source of infection for suckling piglets, and after initial infection, not all animals in the same or different farrows are usually equally affected and therefore vary in severity (Sotiraki et al., 2008).

Typically, after a pre-patenting period of 4–6 days, piglets develop mushy to watery non-hemorrhagic diarrhea, and the most affected animals show reduced weight gain or even weight loss (Nunes et al., 2023).

The age of infection is negatively correlated with the severity of clinical signs and oocyst excretion, with younger animals showing more oocyst excretion and clinical signs (Worliczek et al., 2009).

It has been demonstrated that room temperature has a positive effect on oocyst detection. A one-degree increase in room temperature has been found to increase the likelihood of a litter being positive by 23.2% (Sperling et al., 2022).

The rapid reduction of viable *Cystoisospora suis* oocysts under high temperatures (25 °C and 30 °C) combined with low relative humidity (53% and 62%) leads to oocyst death within 24 h. Viability was higher when oocysts were exposed to higher relative humidity (75% and 100%) and lower temperature (20 °C). However, even at 75% relative humidity, oocysts died within 24–60 h at temperatures ranging from 30 °C to 20 °C, respectively, while the most favorable conditions were 100% relative humidity and 25 °C, where the percentage of viable oocysts decreased from 100% to 17% in 96 h (Langkjær and Roepstorff, 2008).

This study aimed to determine the prevalence and the main intensity of *Cystoisospora suis* and *Cryptosporidium suis* oocysts in fecal samples from suckling piglets in farms in southern and northern districts of Odesa region, to assess the consistency of feces and oocyst release, as well as risk factors associated with the hydrothermal regime of the area.

Materials and methods. From March to December 2023, a total of 818 fecal samples from suckling pigs of the Great White breed were examined in Bolhrad (southern) and Podil (northern) districts of Odesa Region (Fig. 1). The territory of Podil District is located in the forest-steppe zone. Bolhrad District (Bessarabia) is located in the southwestern part of the steppe agroclimatic zone of Ukraine (Adamenko, 2014).

Data on precipitation and average air temperature were obtained from the Bolhrad Meteorological Station (Bolhrad, Odesa Region). Hydrothermal coefficient (HTC) according to the Selyaninov method was used to assess the moisture conditions of the period with average daily temperatures above 10 °C, i.e. the period of active vegetation. Since there are no active air temperatures above 10 °C in some months of the year, HTC was not calculated (Selyaninov, 1937).

HTC was calculated by dividing the amount of precipitation (ΣR) in mm for the period with temperatures above 10 °C by the sum of active temperatures (Σt > 10) for the same period, which was reduced by a factor of 10:

$$HTC = \frac{\Sigma R}{0.1 \times \Sigma t_{act>10}} \quad \text{or} \quad HTC = \frac{\Sigma R \times 10}{\Sigma t_{act>10}}$$

if HTC < 0.4 — very severe drought,
HTC from 0.4 to 0.5 — severe drought,
HTC from 0.6 to 0.7 — moderate drought,
HTC from 0.8 to 0.9 — mild drought,
HTC from 1.0 to 1.5 — sufficiently humid,
HTC > 1.5 — excessively humid.

Fecal samples from piglets of 0–2 months of age were collected directly from the rectum. Samples were taken from 3 to 5 piglets and pooled. Samples were examined at 100× magnification and in doubtful cases at 400× magnification. Samples of unsporulated oocysts were mixed with 2.5% potassium dichromate solution and stored in Petri dishes at 25 °C to induce sporulation.

To determine cryptosporidia for coprological studies, two fecal samples from each animal were prepared on clean, degreased slides. Each sample was examined by making a native smear according to the generally accepted method, staining the smears using the Kester and Romanowski-Giemsa method, followed by microscopy at 630× magnification.

Results. We analyzed the factors of influence of hydrometeorological conditions on the spread of cystoisosporiasis and cryptosporidiosis in piglets.

The characteristics of moisture supply and temperature conditions in Odesa Region according to HTC are shown in Table 1.

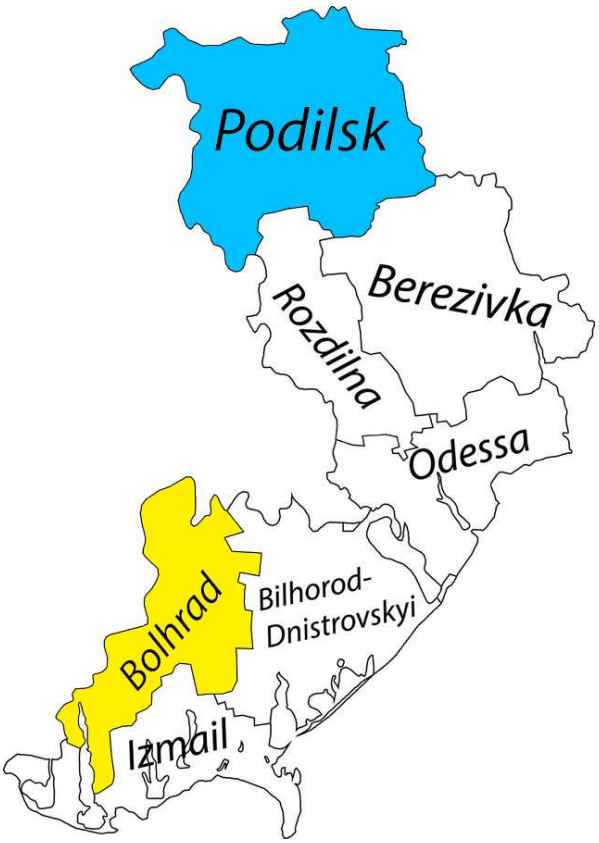

Figure 1. The studied districts of Odesa Region.

Table 1 — Characteristics of hydrometeorological conditions in the southern and northern parts of Odesa Region in 2023

Months	Bolhrad District (southern)				Podil District (northern)			
	Precipitation, mm	Average air temperature, °C	The sum of active temperatures ($t_{act>10}$), °C	HTC	Precipitation, mm	Average air temperature, °C	The sum of active temperatures ($t_{act>10}$), °C	HTC
January	21.1	1.3	0.0	—	31.2	0.8	0.0	—
February	33.5	6.1	0.0	—	45.8	3.1	0.0	—
March	26.4	11.6	360	0.7	42.4	10.9	338	1.3
April	25.1	13.4	402	0.6	52.6	13.2	396	1.3
May	26.2	18.6	577	0.5	65.4	18.7	580	1.1
June	25.5	23.3	699	0.4	42.2	22.8	684	0.6
July	26.7	25.1	778	0.3	50.3	25.0	775	0.6
August	25.8	25.9	803	0.3	60.3	25.7	797	0.8
September	29.7	20.1	603	0.5	58.2	20.0	600	1.0
October	30.1	14.6	453	0.7	59.1	13.5	419	1.4
November	43.4	9.8	0.0	—	49.2	8.7	0.0	—
December	45.1	3.6	0.0	—	51.3	2.8	0.0	—

During four months: January, February, November, and December, HTC was not calculated in both areas because the average daily temperature was below 10 °C.

In Bolhrad District, which is located in the south of Odesa Region, from May to September, HTC ranged from 0.3 to 0.5, indicating very severe to severe drought, and only three months (March, April, and October) were characterized by moderate drought with HTC of 0.6–0.7.

In contrast, in Podil District, which is located in the northern part of Odesa Region, in only two months HTC was 0.6, i.e., moderate drought was recorded, and during five months: March, April, May, September, and October, HTC was in the range of 1.0–1.4 and these months were characterized as sufficiently moist.

HTC influenced the prevalence (P) and the main intensity (MI) of cystoisosporiasis and cryptosporidiosis in suckling piglets (Table 2).

Table 2 — Prevalence and main intensity of cystoisosporiasis and cryptosporidiosis in suckling piglets

Districts	Examined, piglets	Infected, piglets	P, %	*Cystoisospora suis*			*Cryptosporidium suis*		
				Infected, piglets	P, %	MI, oocyst per 10 fields of view of the microscope	Infected, piglets	P, %	MI, oocyst per 10 fields of view of the microscope
Bolhrad	420	145	34,5	116	27,6	32,1±0,5	29	6,9	13,6±0,2
Podil	398	168	42,2	67	16,8	27,2±0,4	101	25,4	19,8±0,6

In the farms of Bolhrad District, the total infection rate with cystoisospores and cryptosporidia was 34.5%, while in the farms of Podil District it was 42.2%.

In the south of the Odesa Region, *Cystoisospora suis* was most often recorded in suckling piglets (27.6%), and in the north — *Cryptosporidium suis* (25.4%). The isolation of oocysts of *Cystoisospora suis* was high in both areas, ranging from 27.2 ± 0.4 to 32.1 ± 0.5 oocysts per 10 microscope fields of view. In comparison, the main intensity of *Cryptosporidium suis* ranged from 13.6 ± 0.2 to 19.8 ± 0.6 oocysts per 10 microscope fields of view.

We discovered the relationship between the feces' consistency and the prevalence of *Cystoisospora suis* and *Cryptosporidium suis* oocysts (Fig. 2).

It was determined that in liquid feces of piglets in both southern and northern districts, cystoisosporiasis was recorded with prevalence of 82.7% and 73.1%, respectively. Moreover, prevalence of piglets with cryptosporidia in the southern area was 37.8% higher than in the northern area. In contrast, in the formed feces of piglets, *Cryptosporidium suis* was most often recorded (72.3%) in the sufficiently humid northern area, while in the southern area, under long-term drought, only 34.5%.

Conclusions. 1. In the farms of the southern part of Odesa Region, which in 2023 was characterized by a very severe drought, *Cystoisospora suis* was most often recorded in suckling pigs (27.6%). Oocysts of *Cystoisospora suis* were found in 82.7% of liquid feces, while in formed feces they were found in 17.3%.

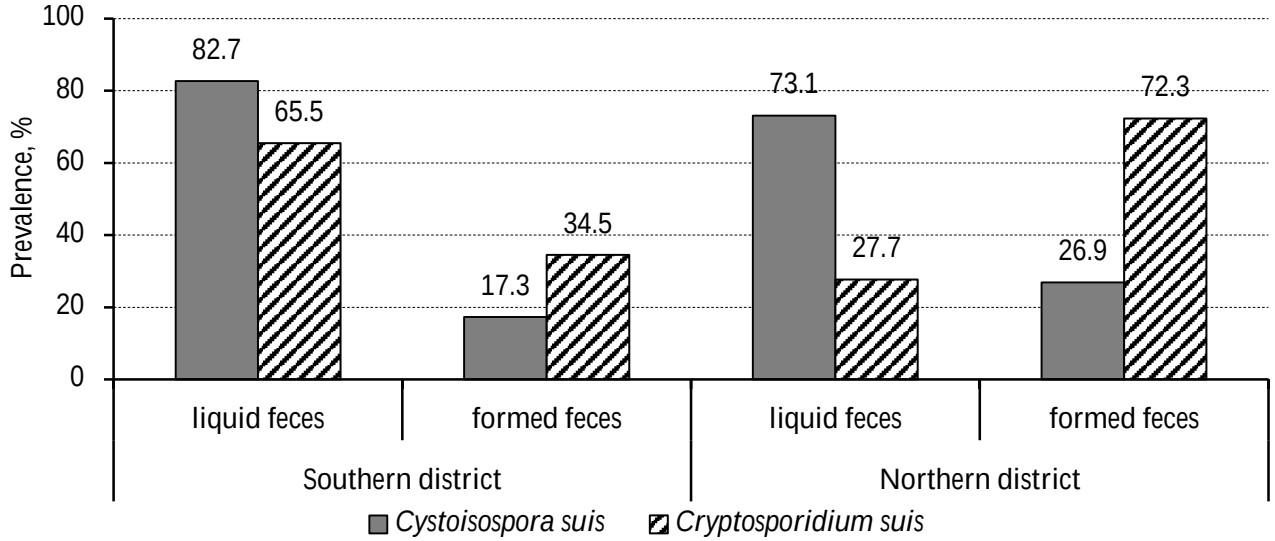

Figure 2. Prevalence of *Cystoisospora suis* and *Cryptosporidium suis* oocysts depending on the feces' consistency.

2. In farms of the northern part of Odesa Region with sufficient moisture, *Cryptosporidium suis* was most often recorded in suckling piglets (25.4%). In the liquid feces of 27.7% of piglets *Cryptosporidium suis* oocysts were detected with the main intensity from 7.3 ± 0.2 to 9.1 ± 0.1 oocysts per 10 microscope fields of view, while in the formed feces of 72.3% of piglets, the main intensity of the infection was from 13.2 ± 0.1 to 16.1 ± 0.1 oocysts per 10 microscope fields of view.

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STUDY THE IMMUNOSTIMULATORY PROPERTIES OF A SOLUTION FOR INJECTION COMPRISING NATURAL POWDERED HONEY IN LABORATORY ANIMALS

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Summary. The article presents the results of pharmacological studies of a new domestic drug 'Apimel' in the form of a solution for injection based on natural powdered honey as an active ingredient. The effect of the solution for injections with natural powdered honey on the severity of the immune response in rats and mice with normal immune status was studied, and the immunostimulating properties of the drug 'Apimel' in mice with immunodeficiency were studied. It was found that the most pronounced immunostimulatory properties of the drug were observed in doses of 50 and 250 mg of natural powdered honey/kg body weight. The study of the effect of the drug on the development of a slow-type hypersensitivity reaction in mice revealed the anti-inflammatory properties of the drug at doses of 25 and 150 mg of natural powdered honey per kilogram of body weight. In the course of studying the immunostimulating properties of the investigated drug in mice with immunodeficiency, it was found that the solution for injection at a dose of 50 mg of natural powdered honey/kg body weight restored the processes of antibody formation at the level of the comparison drug, and significantly exceeded it in terms of the expression of phagocytic activity of neutrophils

Keywords: immunodeficiency, phagocytosis, mice, rats

Introduction. The phagocytic activity of polymorphonuclear leukocytes (PMNs) and reticuloendothelial cells plays a significant role in the body's defense against infection. The capacity of these cells to phagocytize and digest a wide range of microbes that enter the body represents a fundamental protective mechanism of the body (Kuznetsova, Babadzhan and Frolov, 2012). I. I. Mechnikov asserted that the phagocytic reaction is the most significant aspect of natural immunity (Ataman, 2012). It is widely acknowledged that the division of immunity into cellular and humoral components is somewhat arbitrary, given that antibody formation is impossible without the participation of T cells, which are the primary regulators of the immune system. Consequently, the immune response is the result of the cooperative interaction of T and B cells, which to a certain extent reflects the potential capabilities of both immune systems (Kuznetsova, Babadzhan and Frolov, 2012). The antigen processed by the macrophage is recognized by the T helper cell, which involves the B cell in antibody production.

One of the primary methods for maintaining the normal functioning of the immune system and restoring immunity in immunodeficiency states is the use of immunostimulants (Dale, Foreman and Fan, 1994; Babov, Gromov and Nikipelova, 2001; Kasianenko et al., 2020).

These include natural and synthetic substances that can stimulate the body's immune system. Currently, a large number of immunostimulants are used, however, they vary in their effectiveness and many other properties that determine their harmlessness, ease of use, cost-effectiveness, etc. (Butenko et al., 2001).

The most suitable and effective for the body are natural, so-called endogenous immunostimulants, which are based on substances involved in the regulation of immune processes in the human and animal body (Dale, Foreman and Fan, 1994; Bauer et al., 1989). Our country's apiaries produce a natural treasure rich in a full range of vitamins and amino acids, offering a highly effective means for rapid health improvement. Furthermore, the use of bee products for prevention and treatment is not only affordable but also completely natural in composition (Tykhonov et al., 2014).

Honey has been used as a healing agent for thousands of years. Its importance cannot be overstated, as it is a source of nutrients, rich in vitamins B_1, B_2, B_6, E, K, and C, containing folic acid and provitamin A — carotene (Tykhonov et al., 2014).

The well-known cough syrup with plantain and ivy has been shown to have a strong antiviral effect, helping to cope with seasonal acute respiratory viral infections and bronchopulmonary diseases. Thanks to honey, the syrup has a pleasant taste and smell (Kovalenko, 2019).

Experimental studies show that bee honey-based products have a distinct ability to stimulate the phagocytic activity of neutrophils and macrophages, thus increasing nonspecific immunity (Butenko et al., 2001).

It is important to note that special attention should be paid to the substance of natural powdered honey (NPH), the technology of which was developed at the D. P. Salo Department of Pharmacy Technology of Drugs of the National University of Pharmacy (Kharkiv, Ukraine) under the guidance of Prof. O. I. Tikhonov, Academician of the Ukrainian Academy of Sciences (Tykhonov et al.,

2014; Tikhonov, 2010). The substance was used as an active pharmaceutical ingredient to develop a new domestic drug in the form of a solution for injection, which was given the conventional name 'Apimel (Tykhonov et al., 2014).

In light of the above information, it would be beneficial to study the pharmacological properties of the developed drug 'Apimel'.

The study aimed to investigate the effect of a solution for injection with natural powdered honey on the severity of the immune response in rats and mice with normal immune status, as well as to study the immunostimulatory properties of 'Apimel' in mice with immunodeficiency.

Material and methods. Experimental studies were conducted at the Central Research Laboratory of the National University of Pharmacy (Kharkiv, Ukraine).

Experiments on animals were conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and in accordance with Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). The research program was reviewed and approved by the Bioethics Committee of the National University of Pharmacy (Kharkiv, Ukraine) under the current procedure.

During the experiments, the animals were kept in a vivarium at a temperature of 18–24 °C, a humidity of 50–60%, in natural day-night light conditions, in plastic cages, and on a balanced diet under current standards (Kozhemiakin et al., 2002).

To achieve this goal, the effect of the drug on the phagocytic activity of peripheral blood PMNs in rats and the development of a slow-type hypersensitivity reaction in mice was first determined (Freireich et al., 1966).

The ability to influence the phagocytic activity of PMNs was tested by the reaction with yeast.

The investigated solution for injection 'Apimel' (NPH concentration 2.5 wt. %) was administered to rats intramuscularly at doses of 50, 100, 150, and 250 mg NPH/kg body weight for 3 days using five groups of animals:

Group 1 — negative control: rats injected with intramuscular saline;

Groups 2–5: animals injected with NPH solution at doses of 50, 100, 150, and 250 mg NPH/kg body weight, respectively.

To assess the severity of the phagocytic activity of PMNs, the following indicators were calculated: the percentage of polymorphonuclear leukocytes that phagocytosed (Fi — phagocytic index) and the average number of yeast particles absorbed by one PMN (Fu — phagocytic number).

The state of cellular immunity against the background of the experimental drug administration was determined by the delayed-type hypersensitivity reaction (DTH) by the method of Kitamura (1980). This reaction is aimed at determining the ability of the test object to influence the production of mediators by sensitized T effectors that cause tissue infiltration by cellular elements. The injection of antigen into the paw pad of an animal leads to the development of local edema (Kuznetsova, Babadzhan and Frolov, 2012; Kitamura, 1980).

The following groups of animals were used in the experiment:

Group 1 — unimmunized (intact) control;

Group 2 — immunized control, animals that were injected with saline before and during the entire period of immunization with ram erythrocytes (RE);

Groups 3–6 — animals that were injected with 'Apimel' solution at doses of 25, 50, 100, and 150 mg NPH/kg body weight, respectively, before and during the entire period of immunization with RE.

The total period of administration was 6 days.

The animals were immunized via a single intraperitoneal injection of a suspension of freshly washed RE at a dose of 2×10^5 cells in a volume of 0.5 ml of sodium chloride saline per 20 g of body weight. On 5th day, the final dose of antigen (10^8 RE) was administered via injection under the aponeurotic plate of one of the hind limbs (experimental paw) in a volume of 0.02 ml per animal. The same volume of saline was injected into the contralateral paw (control paw). After 24 h, the animals were euthanized by an overdose of ether anesthesia, the feet of the hind limbs were amputated at the level of the tarsometatarsal joint, and they were weighed on a torsion balance.

The severity of the local reaction was assessed by calculating the ratio of the weight of the feet of the experimental and control paws in each group of animals. The reaction index (RI) was calculated using the following formula (1):

$$RI = \frac{M_{exp.paw} - M_{c.paw}}{M_{c.paw}} \times 100\% \quad (1)$$

were: $M_{exp.paw}$ — weight of the experimental paw, g;
$M_{c.paw}$ — weight of the control paw, g.

The experimental data were processed using variation statistics with the standard statistical software package Statistica v. 6.0. To obtain statistical conclusions, the Mann–Whitney test (for data that do not follow the normal distribution law) and parametric methods (Newman–Keuls method) were used. When comparing statistical samples, the significance level of $p < 0.05$ was accepted (Lapach, Chubenko and Babich, 2001).

The next stage of the study was to investigate the immunostimulatory properties of 'Apimel' in mice with immunodeficiency modeled by intraperitoneal single injection of hydrocortisone acetate (Hydrocortison-

Richter, Budapest, Hungary) at a dose of 250 mg/kg body weight (Shvets and Portugalov, 1979).

As a comparison drug, we used a pharmacologically analogous immunostimulant of natural origin 'Thymaline', a lyophilized solution for injection, manufactured by PJSC 'Biofarma' (Kyiv, Ukraine).

The immunostimulatory properties of the test drug were evaluated at doses of 50, 100, and 150 mg NPH/kg body weight. 'Thymaline' was administered at a dose of 100 mg/kg body weight, calculated using the coefficient of conversion of doses by body area from the average daily dose for humans of 10 mg/kg body weight, following the methodology described by Ulanova, Sidorov and Khalepo (1968). The study drugs were administered five days prior to the induction of immunodeficiency and throughout the duration of the experiment, spanning a total of 11 days. One day following the administration of hydrocortisone acetate, mice were immunized with ram erythrocytes to assess the status of the immune system. The antigen was injected intraperitoneally at a dose of 0.2 ml/20 g of body weight.

The following groups of animals were used in the study:

Group 1 — negative control: animals administered drinking water;

Group 2 — pathology control: animals injected with hydrocortisone acetate at a dose of 250 mg/kg body weight;

Groups 3–5 — animals treated with hydrocortisone acetate and injected with natural powdered honey in doses of 50, 100, and 150 mg NPH/kg body weight, respectively;

Group 6 — animals treated with hydrocortisone acetate were administered the comparison drug 'Thymaline' at a dose of 100 mg/kg body weight.

To assess the degree of immunodeficiency and the efficacy of the drugs on 5th day after immunization, animals were euthanized under light anesthesia (chloroform). In the blood serum of experimental animals, the indicators of the humoral immune response titers of hemolysins and hemagglutinins were determined by the method of serial dilutions in polystyrene plates. The agglutination reaction is based on the ability of antibodies (agglutinins) contained in the blood serum of immunized animals to adhere to ram erythrocytes in an isotonic solution of sodium chloride. To evaluate the nonspecific resistance of the mice, we examined the phagocytic activity of neutrophils in the peripheral blood. We calculated the following indicators: the percentage of neutrophils that phagocytosed (Fi — phagocytic index) and the average number of yeast fungi absorbed by one neutrophil (Fu — phagocytic number). To gain further insight into the physiological state of the mice, the weight of the body and organs of immunogenesis (thymus and spleen) was determined as an integral indicator.

The obtained experimental data were statistically processed by the method of variation statistics (arithmetic mean and its standard error (M ± m) or median and upper and lower quartiles (Me (UpQ, LQ;) (M (min; max)). When applying the method of mathematical statistics, the significance level was set at $p < 0.05$. To draw statistical conclusions when comparing statistical samples of relative variables, the Newman–Keuls or Mann–Whitney tests were used (Lapach, Chubenko and Babich, 2001).

Results and discussions. *Investigation of the effect of a solution for injection with natural powdered honey on the phagocytic activity of PMNs.* The study demonstrated the capacity of 'Apimel' to stimulate phagocytosis. The solution exhibited the greatest activity at doses of 50 and 250 mg NPH/kg body weight. The results demonstrated that the drug, administered at a dose of 50 mg NPH/kg body weight, significantly increased the number of absorbed yeast cells compared to the negative control. Furthermore, the phagocytic index, defined as the number of phagocytosed PMNs, increased significantly at a dose of 250 mg NPH/kg body weight (Table 1).

Table 1 — Effect of NPH injection solution on phagocytic activity of rat peripheral blood neutrophils (Me (UpQ; LQ))

Animal groups	Indicators of phagocytosis	
	Fi	Fu
Negative control	41 (29; 46)	3.1 (2.7; 3.4)
'Apimel', 50 mg NPH/kg body weight	45 (43; 55)	3.7 (3.6; 3.8)*
'Apimel', 100 mg NPH/kg body weight	56 (56; 61)*	3.05 (3; 3.1)
'Apimel', 150 mg NPH/kg body weight	50 (39; 54)	3 (2.8; 3.0)
'Apimel', 250 mg NPH/kg body weight	65 (59; 69)*	3.5 (3.2; 3.6)

Note. * — differences are significant relative to the negative control, $p < 0.05$.

It should be noted that in terms of Fu, the activity of the test drug at a dose of 50 mg NPH/kg body weight significantly exceeds that of the drug at doses of 100–250 mg NPH/kg body weight, but in terms of the ability to stimulate PMNs to phagocytosis (phagocytic index), it is inferior to the dose of 250 mg NPH/kg body weight.

Investigation of the effect of a solution for injection with natural powdered honey on the development of a delayed-type hypersensitivity reaction in mice. Administration of 'Apimel' at doses of 50 and 100 mg NPH/kg body weight induced a normal immune response in mice (Table 2).

Table 2 — Effect of NPH solution for injection on the development of delayed-type hypersensitivity reaction in mice (Me (UpQ; LQ))

Animal groups	Dose, mg NPH/kg body weight	Number of animals in a group	RI
Intact (non-immunized) control	—	8	1.7 (0; 3.1)
Immunized control (RE)	—	8	7.2 (3.8; 10.4)*
'Apimel'	25	8	3.7 (1.1; 4.9)**
	50	7	7.4 (4.9; 8.8)*
	100	8	7.5 (3.1; 8.7)*
	150	8	3.5 (0.8; 5.8)**

Notes: * — differences are significant relative to the intact control, $p < 0.05$; ** — differences are significant compared to the immunized control, $p < 0.05$.

The study established the ability of the drug to suppress the development of an immune inflammatory response at doses of 25 and 150 mg NPH/kg body weight, as indicated by significantly lower values of the corresponding DTH reaction index.

Investigation of immunostimulatory properties of the solution for injections with natural powdered honey in mice with immunodeficiency. The data obtained in previous studies on the immunostimulatory effect of 'Apimel' solution for injection on animals with normal immune status served as a basis for determining the effectiveness of the drug in conditions of immunodeficiency in mice modeled by hydrocortisone acetate (Tables 3–5).

Table 3 — Effect of NPH solution for injection on phagocytic activity of peripheral blood neutrophils in mice with immunodeficiency modeled by hydrocortisone acetate (Me (UpQ; LQ))

Animal groups	n	Indicators	
		Fi	Fu
Negative control	11	25 (21; 28)	3.4 (2.4; 3.6)
Control pathology	10	17 (15; 18)*	3.3 (3.0; 3.8)
'Apimel', 50 mg NPH/kg body weight	11	28 (26; 33) **/***	3.5 (3.3; 4.3)
'Apimel', 100 mg NPH/kg body weight	10	15 (10; 26)#	3.3 (3.1; 3.9)
'Apimel', 150 mg NPH/kg body weight	10	17 (15; 21)*#	3.2 (2.1; 4.8)
'Thymalin', 100 mg/kg body weight	8	21 (17; 23)	3.8 (3.2; 5.1)

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology, $p < 0.05$; *** — differences are significant in relation to the values of the comparison drug 'Thymaline', $p < 0.05$; # — differences are significant in relation to the values of the experimental drug at a dose of 50 mg NPH/kg body weight, $p < 0.05$.

Table 4 — Effect of NPH solution for injection on the indices of humoral immunity in mice with immunodeficiency modeled by hydrocortisone acetate (Me (UpQ; LQ)

Animal groups	n	Indicators	
		Hemolysins, log_2	Hemagglutinins, log_2
Negative control	11	7 (5; 9)	8 (7; 8)
Control pathology	10	1.5 (0; 3)*	1 (0; 3)*
'Apimel', 50 mg NPH/kg body weight	11	5 (3; 5)**	4 (3; 6)**
'Apimel', 100 mg NPH/kg body weight	10	4 (3;5)**	4 (3; 4)**
'Apimel', 150 mg NPH/kg body weight	10	4 (2;5)*/**	3 (3; 4)*/**
'Thymalin', 100 mg/kg body weight	8	5 (4; 5.5)**	3.5 (3; 4.5) */**

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology.

Table 5 — Effect of NPH solution for injection on the dynamics of mass coefficients of mice organs under conditions of immunodeficiency modeled by hydrocortisone acetate ($M \pm m$)

Animal groups	n	Weight, g	Mass coefficients of the organs	
			Thymus	Spleen
Negative control	11	22.3 ± 0.55	0.32 ± 0.03	0.91 ± 0.05
Control pathology	10	17.8 ± 0.57*	0.13 ± 0.013*	0.48 ± 0.17*
'Apimel', 50 mg NPH/kg body weight	11	17.7 ± 0.54*	0.23 ± 0.02*/**	0.29 ± 0.03*
'Apimel', 100 mg NPH/kg body weight	10	18.6 ± 0.54*	0.16 ± 0.012*	0.31 ± 0.03*
'Apimel', 150 mg NPH/kg body weight	10	18.7 ± 0.45*	0.18 ± 0.02*	0.38 ± 0.06*
'Thymalin', 100 mg/kg body weight	8	17.6 ± 0.34*	0.20 ± 0.02*	0.27 ± 0.03*

Notes: * — differences are significant relative to the values of the negative control, $p < 0.05$; ** — differences are significant relative to the values of control pathology.

The administration of hydrocortisone acetate to mice of the control pathology group led to the development of immunodeficiency, which was reflected in impaired

phagocytosis and decreased antibody production and was accompanied by a decrease in both body weight and mass coefficients of the thymus and spleen of mice, which is a natural result of the cytotoxic effect of high doses of hydrocortisone acetate.

The results of the study on the effect of the solution for injection with natural powdered honey on the phagocytic activity of polymorphonuclear leukocytes of peripheral blood of rats and the development of a delayed-type hypersensitivity reaction in mice indicate that the most pronounced stimulating properties of the drug 'Apimel' are found in doses of 50 and 250 mg NPH/kg body weight. Upon analysis of the data from the study on the effect of the solution for injection with natural powdered honey on the development of a delayed-type hypersensitivity reaction in mice, it was determined that 'Apimel' in doses of 25 and 150 mg NPH/kg body weight suppresses the immune inflammatory reaction provoked by the introduction of RE, indicating the anti-inflammatory properties of the drug. At doses of 50 and 100 mg NPH/kg body weight, the solution for injection did not affect the normal immune response of mice to antigen administration.

A study of the immunostimulatory properties of the solution for injections with NPH in mice with immunodeficiency revealed a notable decline in the phagocytic activity of peripheral blood neutrophils. The number of phagocytic neutrophils was found to be 1.5 times lower than that of the negative control (Drogovoz et al., 2010). However, the phagocytic number remained at the level of the negative control (Table 3). At the same time, the process of antibody formation was impaired, as evidenced by a significant decrease in the titers of hemolysins and hemagglutinins in the serum of animals by 78 and 88%, respectively (Table 4).

The administration of hydrocortisone acetate to mice resulted in the development of secondary immunodeficiency, characterized by impaired nonspecific resistance and antibody formation in animals. Conversely, prophylactic intramuscular injection of the test drug 'Apimel' led to the restoration of animal immunoreactivity, with hemolysins' and hemagglutinins' titers exceeding those of the control group by an average of 3–4 times. The solution for injection with NPH demonstrated the most pronounced effect at a dose of 50 mg NPH/kg body weight. In animals injected with the drug at a dose of 50 mg NPH/kg body weight, both antibody production and the phagocytic activity of neutrophils were increased to the level of the negative control (Tables 3, 4). Furthermore, at a dose of 50 mg NPH/kg body weight, the drug demonstrated a unique effect of increasing thymic mass coefficient, in contrast to the other experimental groups (Table 5). Meanwhile, spleen mass coefficient remained significantly lower than the values observed in the negative control group (Jerne and Nordin, 1963).

It is important to highlight that the 'Apimel' solution for injection, at a dose of 50 mg NPH/kg body weight, demonstrated comparable efficacy to the reference drug in restoring antibody formation processes. However, it significantly exceeded the reference drug in terms of the phagocytic activity of neutrophils (Table 3). Our studies have proven the potential of 'Apimel' solution for injection as a viable treatment option.

Conclusions. The solution for injection with natural powdered honey, when administered intramuscularly to rats in the dose range of 50–250 mg NPH/kg body weight, has been observed to demonstrate moderate phagocytic activity.

'Apimel' has been shown to stimulate the phagocytic activity of PMNs most effectively at doses of 50 and 250 mg NPH/kg body weight. At doses of 25 and 150 mg NPH/kg body weight, the test drug demonstrated efficacy in suppressing the development of the DTH reaction in mice, indicating the anti-inflammatory properties of the drug.

The solution for injection with natural powdered honey exhibited notable immunostimulatory properties in conditions of immunodeficiency induced by hydrocortisone acetate. Its efficacy was comparable to that of 'Thymalin', a well-known immunomodulator of natural origin.

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Part 2. Biotechnology

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IDENTIFICATION OF INTRAMOLECULAR CONSERVED G-QUADRUPLEX MOTIFS IN THE GENOME OF THE BOVINE FOAMY VIRUS

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Summary. G-quadruplexes (G4s) are guanine-rich DNA structures, which play an essential regulatory role in key steps of the viral life cycle (replication, transcription regulation, translation). Currently, there is no relevant information about putative G4s in the bovine foamy virus (BFV) genome. The goal of the present study was the determination of such conservative non-B-DNA structures as conservative G-quadruplexes, which can be formed by two and three G-quartets in the mRNA, sense, and antisense strands of the bovine foamy virus proviral DNA. Bioinformatic analysis was used to search motifs of intramolecular G-quadruplexes in BFV mRNA and proviral DNA and to determine the G-score (a parameter that characterizes the stability of the G-quadruplex in relative units). Based on multiple alignments of 27 BFV isolates 26 putative conservative G-quadruplexes from two G-quartets were found in mRNA and sense strand of BFV proviral DNA with G-score from 30 to 36. 32 G4s formed by two G-quartets with a G-score from 30 to 36 and 2 G4s formed by three G-quartets were found in the antisense strand of BFV proviral DNA with a G-score of 53. These two G4s are direct repeats and are localized in U5 5'LTR and U5 3'-LTR. The density of G4s was 2.1/kbp in the sense strand of BFV proviral DNA and 2.8/kbp in the antisense strand. A localization map of potential stable conserved intramolecular G-quadruplexes formed by two and three G-tetrads on the BFV genome was created. Conservative G4s are unevenly distributed throughout the BFV genome. A distinctive feature of the BFV genomic organization is the fact that the antisense strand of the BFV proviral DNA is characterized by a significantly higher density of G-quadruplexes compared to one of the sense strands. The QGRS Mapper software detects a significantly higher number of potential G4s (34 G4s in the antisense strand of BFV proviral DNA) compared to the G4Hunter software (7 G4s)

Keywords: bovine foamy virus, BFV, G-quadruplex, motif, non-canonical structure, direct repeat, antisense strand

Introduction. Foamy viruses (or spumaviruses) belong to the subfamily Spumaretrovirinae of the family Reoviridae. They were found in many species of animals, including cattle, and they are the most ancient among all known retroviruses (Pinto-Santini, Stenbak and Linial, 2017; Rethwilm and Bodem, 2013). The first foamy virus was described in 1954 (Enders and Peebles, 1954). However, the first bovine foamy virus (BFV), which belongs to the genus *Bovis foamy virus*, was isolated later (Malmquist, Van der Maaten and Boothe, 1969) and since then BFV-seropositive animals have been detected in many countries of the world.

The BFV infection rate for different countries varies considerably. In Canada, it ranged from 40 to 50%. A slightly lower level (39%) was observed in Australia and Great Britain. In Germany, only 7% of tested animals were identified as BFV-seropositive, in Poland among dairy cattle its value was 30%. In Japan, the infection level for different prefectures ranged from 12 to 16%. The highest level of seropositivity was typical for age-related animals (Materniak-Kornas et al., 2019; Okamoto et al., 2020).

Clinical symptoms or diseases associated with BFV have not yet been described, and the virus is considered a non-pathogenic one (Meiering and Maxine, 2001). However, the possibility of mixed infection with bovine leukemia virus, bovine immunodeficiency virus, and foamy virus, considered cofactors, has been established (Materniak-Kornas et al., 2017; Le et al., 2021).

BFV mRNA with a length of about 12.0 kb contains three typical for retroviruses structural *gag*, *pol*, *env* genes and two additional non-structural characteristics for foamy viruses' *bel-1* and *bel-2* genes, which are localized between *env* gene and 3'LTR and encode Bet protein. This protein consists of Tas and Bet2 parts. Unlike the Tas protein, which is a transcriptional activator, the function of the Bet2 protein has not yet been established (Jaguva Vasudevan et al., 2021; Mekata et al., 2021).

The nucleic acid molecules can form non-canonical structures (triplexes, hairpin structures, i-motifs (intercalated

motifs), G-quadruplexes, etc.) that are regulatory elements. Triplexes can regulate gene expression, and participate in chromatin organization and DNA recombination (Jenjaroenpun and Kuznetsov, 2009; Dalla Pozza et al., 2022). RNA hairpin structures called RNA thermometers control and regulate a variety of processes in microorganisms at the translational and transcriptional levels (Abduljalil, 2018). The possibility of the crucial role of i-motif in protein synthesis, chromosome integrity, and mitosis regulation is discussed (Luo et al., 2023).

G-quadruplexes (G4) are formed by planar G-quartets in DNA or RNA guanine-rich sequences under physiological conditions. G-quadruplexes are stabilized by Hoogsteen hydrogen bonds, G-quartets stacking, and monovalent cations K^+, and Na^+. G-quadruplexes are composed of one (intramolecular G4) (Fig. 1), and two or four (intermolecular G4) nucleic acid strands. G-quartets in intra-molecular G4 are connected by different loops with lengths of 1–12 nt and are influenced by G-quadruplexes' stability (Zaccaria and Fonseca Guerra, 2018).

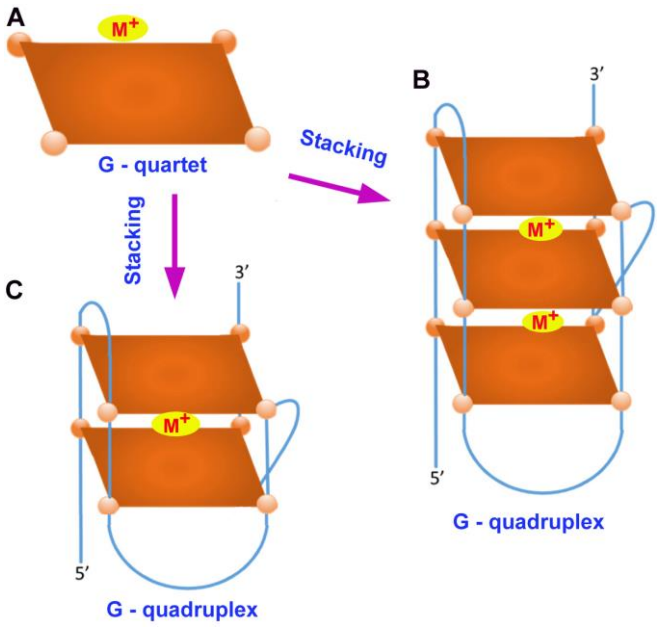

Figure 1. G-quartet involves four coplanar guanines establishing a cyclic array of H-bonds (A). Intramolecular anti-parallel G-quadruplex structure formed by stacking of three (B) and two (C) G-quartets and stabilized by monovalent cations, such as potassium, ammonium, and sodium (Brázda et al., 2020).

G-quadruplexes play significant roles in different biological processes such as translation, transcription, recombination, replication, etc. and they are found in micro- and macroorganisms. G-quadruplexes are associated with some human diseases and are considered prospective targets in molecular medicine (Cagirici, Budak and Sen, 2021; Harris and Merrick, 2015; Perrone et al., 2017). To predict G4 structure formation several methods have been developed (Puig Lombardi and Londoño-Vallejo, 2020).

There are spectroscopic techniques and biochemical methods for G4 identification, visualization, and function determination (Umar et al., 2019).

Regarding the formation of non-canonical structures in the foamy virus genome, information is very limited. The existence of a hairpin structure with the possibility of a stable pseudotriloop structure formation in the genomic RNA of the human foamy virus is shown by NMR spectroscopy and UV melting. This pseudotriloop structure is conserved among all known spumaretroviruses, including BFV, and it may be a recognition sites for proteins (Van Der Werf et al., 2013). As for the existence of motifs with the potential to form G-quadruplexes in the genome of foamy viruses, there is currently no relevant information in the literature.

Aim. In this study, putative conservative G-quadruplexes, which can be formed by two and three G-quartets in the mRNA, sense, and antisense strands of the bovine foamy virus proviral DNA was determined based on the 27 BFV isolates nucleotide sequences with a complete genome.

Materials and methods. Nucleotide sequences of 27 BFV isolates were obtained by searching taxonomic identifier (txid) 207343 in the GenBank database of the National Center for Biotechnology Information (USA).

BioEdit software (version 7.2.5) (Hall, 1999) was used to obtain a consensus sequence of BFV with a complete genome. The search for GGG motifs in the BFV mRNA and proviral DNA, manipulation with nucleotide sequences (reverse, complement), and multiple alignments were performed using the Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.06) (Tamura et al., 2013).

QGRS Mapper software on the web server https://bioinformatics.ramapo.edu/QGRS/index.php (Menendez, Frees and Bagga, 2012; Kikin, D'Antonio and Bagga, 2006) was used for searching motifs of intramolecular G-quadruplexes in mRNA, sense, and antisense strands of the BFV proviral DNA, as well as to determine the G-score (a parameter that characterizes the stability of the G-quadruplex in relative units) with the following parameters: the maximum length of the G-quadruplex is 45 nt, the minimum size of the G-quartet number is 2, the loop — from 0 to 20 nt. Additionally, putative G-quadruplexes were identified by G4Hunter software on the web server https://bioinformatics.ibp.cz (Bedrat, Lacroix and Mergny, 2016; Brázda et al., 2019) with a window of 25, the threshold of 1.2.

Results. G-quadruplexes in DNA and RNA have different topologies. The topology of RNA G4s is limited to the parallel conformation where all four strands are oriented in the same direction. In contrast, DNA G4s can adapt to parallel, antiparallel, or mixed conformations.

Intramolecular interactions within RNA G4s lead to enhanced stability of RNA G4s compared to DNA G4s (Fay, Lyons and Ivanov, 2017).

Sequences forming G-quadruplexes were used as a control of the correctness of the search for G4s motifs. These G4s were experimentally confirmed for the hepatitis B virus (HBV) and found in the genome of tobacco (*Nicotiana tabacum*), which is an ideal plant model in scientific research. The HBV genome is represented by double-stranded and relaxed circular DNA with a length of approximately 3,200 bp. 13 G4s were found in the HBV genome, for the seven of which the determined G-score value was ranged from 19 to 35 (Table 1). For the five G4s most frequently found in the tobacco genome, the G-score varied from 20 to 29 (Table 1).

Table 1 — Conservative G-quadruplex motifs which were determined and used as a control for determining the G-score. G-rich fragments of sequences for putative G-quadruplexes are underlined. Positions of G4s on the HBV genome and the number of G4s in all chromosomes of the tobacco (*Nicotiana tabacum*) nuclear genome are shown in parentheses

Virus/Plant	Length, nt	G-quadruplex motif	G-score
HBV (1207)	19	GGCUGGGGCUUGGUCAUGG (Wang et al, 2023)	35
HBV (1739)	22	GGAGUUGGGGGAGGAGAUUAGG (Wang et al, 2023)	34
HBV (1889)	19	GGGUGGCUUUGGGGCAUGG (Wang et al, 2023)	33
HBV (2992)	23	GGAGCUGGAGCAUUCGGGCUGGG (Wang et al, 2023)	33
HBV (3034)	25	GGCCUUUUGGGGUGGAGCCCUCAGG (Wang et al, 2023)	31
HBV (261)	28	GGUGGACUUCUCUCAAUUUUCUAGGGGGG (Wang et al, 2023)	19
HBV (1779)	22	GGAGGCUGUAGGCAUAAAUUGG (Wang et al, 2023)	29
Tobacco (1392)	24	GGGGGTGTGTACAGACTCCGGAGG (Song et al, 2024)	23
Tobacco (605)	22	GGGGGCCTCGGGTGTGTTTCGG (Song et al, 2024)	29
Tobacco (587)	23	GGGGTGTGTACAGACTCCGGAGG (Song et al, 2024)	22
Tobacco (599)	26	GGGGGGTTGACTTTTTGATATCGGGGG (Song et al, 2024)	20
Tobacco (575)	22	GGGGGTGTACAGACTCCGGAGG (Song et al, 2024)	25

The sense strand, or coding strand, is a strand within double-stranded DNA that carries information about the translation code in the 5' to 3' direction. Its complementary strand is called the antisense strand, which does not carry a translation code and serves as a matrix during translation. The sense strand of DNA has the same sequence as mRNA, which contains codon sequences for

Taking into account the experimental data on the parameters of the detected G4s in the genome of HBV and tobacco (Table 1), only conservative fragments were analyzed for BFV isolates, which can form G-quadruplexes with G-score ≥ 30. $G_xN_yG_xN_yG_xN_yG_x$ motif with a maximum length of 45 nt and $2 \le x \le 4$, $1 \le y \le 10$ was used to predict putative G4s (Kikin, D'Antonio and Bagga, 2006).

G4s can affect gene expression also if they are localized on the sense strand concerning the direction of transcription. For this reason, the search of putative G4s motifs was performed both for sense and antisense strands of the BFV proviral DNA.

Based on multiple alignment of 27 BFV isolates 26 putative conservative G-quadruplexes from two G-quartets were found in mRNA and sense strand of BFV proviral DNA with G-scores from 30 to 36 (Table 2). 32 G4s formed by two G-quartets with a G-score from 30 to 36 and 2 G4s formed by three G-quartets were found in the antisense strand of BFV proviral DNA with a G- score of 53 (Table 3). These two G4s are direct repeats and they are localized in 5'- and 3'-LTR. The density of G4s in the sense strand of BFV proviral DNA was 2.1/kbp, and the density of G4s in the antisense strand was 2.8/kbp.

A comparison of the G4s search effectiveness by two software — QGRS Mapper and G4Hunter — was carried out. 26 putative G4s for mRNA and the sense strand of BFV proviral DNA (Table 2) were identified by QGRS Mapper software, while no G4s were identified by G4Hunter. 34 putative G4s were identified by QGRS Mapper software for antisense strand of BFV proviral DNA, while seven G4s were identified by G4Hunter software (Table 3, motifs are highlighted), six of which are fragments of the G4s identified by QGRS Mapper software.

The formation of non-B-DNA structures requires the unwinding of double-stranded DNA molecules, which occurs during replication and transcription. It is known that G4s in the antisense strand significantly inhibit transcription, unlike G4s in the sense strand, which do not affect transcription (Agarwal et al., 2014). Computer

analysis of 27 BFV isolates with a complete genome showed that G4s are unevenly distributed both among structural genes and in the sense and antisense strands of BFV proviral DNA.

Table 2 — Conservative sequences for putative G-quadruplexes in mRNA and sense strand of bovine foamy virus proviral DNA based on multiple alignments of 27 BFV isolates. G-rich fragments of sequences for putative G4s are underlined. Gene positions are indicated for JX307861 BFV isolate. DR3, DR4, DR5 are the direct repeats

Position	Gene/region	G-quadruplex motif		G-score
56	U3	GGAGGATTGGCTGG	DR3	34
10754	U3	GGAGGATTGGCTGG	DR3	34
486	U3	GGTTCGGAGGATGG	*DR4*	34
11184	U3	GGTTCGGAGGATGG	*DR4*	34
574	U3	GGTTCGAGGTCAGGCGG	DR5	32
11272	U3	GGTTCGAGGTCAGGCGG	DR5	32
1716	*gag*	GGtatctagatatGGtcccttagaagggGGtgattatcagccaGG		34
2183	*gag*	GGCATCCCGGGCTCGCCTTGGAACCCTTGG		35
2914	*gag*	GGaagaagcagccaaGGaacaaacaacacaGGaagttctgcCGG		32
3031	*gag*	GGaaatcaagGGCaatcatcttaaaGGCTACTGGGACTCTGG		31
3616	*pol*	GGCTGATGGGCGCTGGAGGATGG		36
6225	*pol*	GGactcccagtcctGGcctgttggtccaGGAGAGGGTAGCCAGG		36
6582	*pol*	GGCATCGTTGGAAGGAGTGCGGG		31
7309	*env*	GGcactgttacgtGGTACATATTCCGGGGGGAAAAGAATGG		36
7688	*env*	GGCACTTCCAGGAACCTGGCACACTCTAGG		32
8770	*env*	GGATCCGAAACTGGCAAGTGGCCAATCTAGG		31
8939	*env*	GGTGGTGGAAGTGGAACCTTGTGGCAACCTGACAGG		34
9332	*env*	GGatctccacgaaGGAagaattactcagactGGatcctgcagcttGG		33
9429	*env*	GGGACCTTACTGGAGAAAGCTGCAGGAACTCTCTTCGG		34
10013	*bel1*	GGAGGAGGAACACCCGG		30
10043	*bel1*	GGTGGAGACCAGGACATGCGGGTCAATACTTCCGG		32
10239	*bel1*	GGTTTTGGACAGGTGATGATAAAGGCCCACAGGAATGG		34
11002	U3	GGCCAAGCAGGCCTCCCGGCGGG		31
11002	U3	GGCCAAGCAGGCCTCCCGGCGGG		31
11272	R	GGTTCGAGGTCAGGCGG		32
11604	U5	GGggaattattgGGaatcccatattgtaGGAAAAGTCAGTTGG		33

We have indicated the sites of putative G4s on the BFV genomic map, which shows the relative positions of structural genes, regulatory elements, genetic markers, and the distance between them (Fig. 2). 4, 3, and 7 G4s were identified in *gag*, *pol* and *env* genes of the sense strand, while 10, 11, and 5 G4s were identified for the above-mentioned genes of the antisense strand, respectively. Represented data indicate that the formation of G4s in sense and antisense strands of BFV proviral DNA occurs asymmetrically, which may result in different effects of G4s, which are identified in sense and antisense strands, on the transcription.

Designed genomic map of putative G4s sites has permitted to reveal the features of the structural organization of the sense strand in comparison to the antisense strand of BFV proviral DNA. Three direct repeats (DR3–DR5) formed by two G-tetrads with G-score from 32 to 34 were found in 5'- and 3'-LTR of the sense strand. Two direct repeats (DR1–DR2) were found in 5'- and 3'-LTR of the antisense strand, one of which (DR1) is formed by two G-tetrads (G-score equals 32). Another direct repeat (DR2) is formed by three G tetrads, and the G-score of G4s is 53.

Table 3 — Conservative sequences for putative G-quadruplexes in the antisense strand of bovine foamy virus proviral DNA based on multiple alignments of 27 BFV isolates. G-rich fragments of sequences for putative G4s are underlined. Gene positions are indicated for JX307861 BFV isolate. W=A/T, Y=C/T. DR1 and DR2 are the direct repeats. Identified by G4Hunter software motifs with corresponding G-score values in parenthesses are highlighted

Position	Gene/region	G-quadruplex motif		G-score
11888	U5	GGaaggtaagcccaGGttggagtcaGGttcccactcgcacGG	*DR2*	32
1190	U5	GGaaggtaagcccaGGttggagtcaGGttcccactcgcacGG	*DR2*	32
10715	U3	GGGCAAGTAAAGGGGGGCTGGccyttgctwatttcyGGG	DR1	53
17	U3	GGGCAAGTAAAGGGGGGCTGGccyttgctwatttcyGGG	DR1	53
10671	*bel2*	GGAGGGCATGGCTGG		35
10092	*bel1*	GGTCATCCCTGGCACATTGGCACAAAGAGG		34
9973	*bel1*	GGGGTGGAGG (35)		35
9915	*bel1*	GGTTTTGTCCTAGGATTGCATCGATCGGTCAGATGCCTGG		34
8234	*env*	GGAGGGAGAAGGCTCGG		34
7676	*env*	GGTTCCTGGAAGTGCCTCAGGGTTAAGG		31
7561	*env*	GGAGTATAGGGTAGGGCTACAGAAGG		32
7061	*env*	GGGTTTTGGGTGAGGTTCCAGG		35
6668	*env*	GGTTCATCTGCAGGAGGTTGGTCGTCTCTAGG		31
6222	*pol*	GGCtaccctctcctGGaccaacaggccaGGACTGGGAGTCCAGG		36
6193	*pol*	GGGCAGTGGTCTGTGAAGGAGAAGGG		33
5906	*pol*	GGGGGTGGTAAGG		34
5830	*pol*	GGAGGAGGTGAATGCTGGCCCCTGG		32
5743	*pol*	GGTTGCGTTGGCAGTTTGGGCCTTGGTGGGG (33)		36
5669	*pol*	GGAGGGAGGGAGTGG		35
5165	*pol*	GGTTGGTGGCCTGG		34
4250	*pol*	GGATACATTGGCCTTTGGCTTTGGG		34
3844	*pol*	GGAGAGTTTAGGAATCCCTGGGGGAGG		32
3254	*pol*	GGTCCTGGTTTATACCAGGGAATATCGATAGG		30
2986	*pol*	GGCGGAGTGCCGGCATCTTGTGGGGGTATGGGTGG (34)		36
2874	*gag*	GGGGGCGGGACCTCGG		32
2818	*gag*	GGGGTTGGTTGTCGATAAGGGTTCGG		30
2746	*gag*	GGATTGGGCCTGAGGGGATAACGAGG (31)		33
1987–2006	*gag*	GGCCCTGGAGCAGGTGCAGG		36
1969–1979	*gag*	GGTGGAGGAGG		36
1939–1958	*gag*	GGAGCAGGCAATGGAGCTGG		36
1915–1932	*gag*	GGCTGGAGGTGGTGGAGG (36)		35
1891–1918	*gag*	GGAGGAGCAGGTCCTTGTGGAGCCGCTGG		36
1633	*gag*	GGGTAAGGTTGGGTCCGAGG		34
1573	*gag*	GGTTCCCATTGAGGAATAGGCAAGGGTTGCCCTGCAGG		36

Structural genes of the sense strand are characterized by a different density of G4s compared to one of the antisense strands of BFV proviral DNA. For the *gag* gene of the sense strand, the density of G4s was 2.5/kbp, while one for the antisense strand was 6.3/kbp. For the *pol* gene of the sense strand the density of G4s was 0.8/kbp, while one for the antisense strand was 3.0/kbp. For the *env* gene of the sense strand, the density of G4s was 2.4/kbp, while one for the antisense strand was 1.7/kbp.

The antisense strand of BFV proviral DNA is characterized by an extremely high density of G4s for distinct gene fragments, which is not observed for the

sense strand. In particular, in the fragment of the *pol* gene with a length of 116 nt (positions 1891–2006 nt) 5 G4s were identified and the density of G4s was 43.1/kbp. For the longer *pol* gene fragment (406 nt, positions 1891–2006 nt), taking into account the additional two detected G4s, the density of G4s was 16.1/kbp.

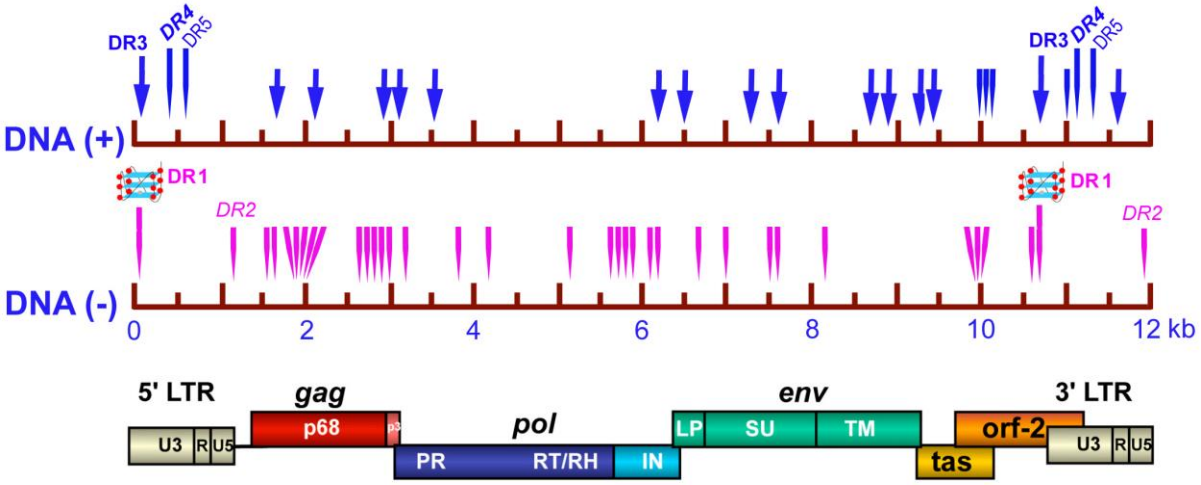

Figure 2. Genomic map of the bovine foamy virus genome with the positions of known genes (Hamann and Lindemann, 2016). Arrows show sites of G-quadruplex motifs in sense and antisense strands of BFV proviral DNA. Small G4 models indicate putative intramolecular G-quadruplexes whose sequences are direct repeats at the 5'- and 3'-ends of the antisense strand of proviral DNA with a G-score of 53.

Conclusions. In the present study, the existence of conservative motifs in mRNA, sense, and antisense strands of BFV proviral DNA, which contain guanine-riched fragments with the potential to form G-quadruplexes was demonstrated. Comparison of the G-score for experimentally determined known from the literature G4s and ones for BFV shows that the G-score for putative G-quadruplexes in the BFV genome (30–53) coincides with the G-score for the hepatitis B virus, *Nicotiana tabacum*.

Conservative G4s are unevenly distributed throughout the BFV genome. A distinctive feature of the BFV genomic organization is the fact that the antisense strand of the BFV proviral DNA is characterized by a significantly higher density of G4-quadruplexes compared to one of the sense strands. The QGRS Mapper program detects a significantly higher number of putative G4s (34 G4s in the antisense strand of BFV proviral DNA) compared to the G4Hunter program (7 G4s).

Represented data indicate that the formation of G4s in sense and antisense strands of BFV proviral DNA occurs asymmetrically, which may result in different effects of G4s on the transcription. Structural genes of the sense strand are characterized by a different density of G4s in comparison to one of the antisense strands of BFV proviral DNA.

A designed genomic map of the distribution of putative G4s raises several questions because the biological function of the vast majority of G4s is uncertain. It is known that G4s, which are localized in the 5'- and 3'-LTR, have different effects on translation (Kikin, D'Antonio and Bagga, 2006). G4s in the antisense strand create blockage for RNAPII unlike G4s in the sense strand and thus reduce transcription (Agarwal et al., 2014).

The identification in the 5'- and 3'-LTR of three direct repeats formed by two G-tetrads for the sense strand and two direct repeats, one of which is formed by three G-tetrads, for the antisense strand indicates that G4s, which are localized in the 5'- and 3'-LTR of the sense and antisense strands, can have different effects on transcription and translation.

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INVESTIGATING THE STABILITY OF A SYMBIOTIC BIOLOGICALLY ACTIVE SUPPLEMENT FOR ANIMALS DURING STORAGE

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Summary. The aim of this work was to determine the stability of a symbiotic biologically active supplement for animals during storage and to establish its shelf life. The stability of the symbiotic was studied under two temperature conditions. The results obtained indicate that the symbiotic biologically active supplement for animals retained its biochemical activity on the day of manufacture and after 3, 6, 9, 12, 15, 18, 21, and 24 months and was harmless to white mice throughout the study period when stored at a temperature of 4 ± 0.5 °C. The drug remained suitable for use for 3 months after manufacture when stored at room temperature (20 ± 2 °C). Taking into account the results of the experimental data, it is recommended to store the symbiotic in the refrigerator at a temperature of 4 ± 0.5 °C for 24 months and at a temperature of 20 ± 2 °C for 3 months

Keywords: *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, quality indicators

Introduction. Preventing and treating gastrointestinal diseases in animals remains a significant challenge in modern veterinary medicine (Vovk et al., 2021; Kolechko et al., 2023; Cherevan et al., 2018). Recently, complex biological drugs based on probiotics and prebiotics, called symbiotics, have been employed in animal husbandry (Gujvinska and Paliy, 2018; Iegorov, Kananykhina and Turpurova, 2021; Mizernytskyi, 2021). The International Scientific Association for Probiotics and Prebiotics (ISAPP) has defined a symbiotic as a mixture containing living microorganisms and substrates selectively used by host microorganisms that are beneficial to the health of the host (Swanson et al., 2020). It should be noted that many probiotics, prebiotics, and symbiotics have been produced recently (AEProbio, 2021; Gibson et al., 2017; Hardy, 2023; Roberfroid, 2000; Taboada et al., 2022; Tavaria, 2017; Gujvinska, 2015; Solovyova and Kaliuzhnaia, 2021).

However, the existing literature contains a lack of information on studies of symbiotic stability. Consequently, the manufacturer's responsibilities include the study of product stability, the results of which determine the shelf life and storage conditions (MHU, 2004). The shelf life and storage conditions of active substances and medicinal products can only be determined based on the results of their stability studies. It should be noted that drug stability studies should be carried out at the stage of development of active substances and medicinal products, as well as during storage.

The study aimed to determine the stability of a symbiotic biologically active supplement for animals during storage.

Materials and methods. The stability of the symbiotic biologically active supplement for animals during storage was studied in the Laboratory of Veterinary Sanitation, Parasitology and Bee Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine). Six pilot batches of the symbiotic biologically active supplement were manufactured and its stability during storage was studied.

In order to study the stability of the symbiotic biologically active supplement during storage, we conducted several scientific experiments. The shelf life of the symbiotic biologically active supplement was determined by the quality of the symbiotic, as well as by the indicators of microbiological purity (bacterioscopic control and absence of extraneous microflora), harmlessness, specific activity (the number of live bacteria in one dose of the drug).

The control parameters were determined at different storage periods of the biological product, namely on the day of its manufacture and during storage for 3, 6, 9, 12, 15, 18, 21, and 24 months. For this purpose, an archive of 18 drug samples was formed. The symbiotic biologically active supplement for animals was stored at room temperature (20 ± 2 °C) and in a refrigerator at a temperature of 4 ± 0.5 °C. To evaluate the quality of the archived samples of the symbiotic biologically active supplement for animals, three samples of the drug were taken after a certain shelf life.

The symbiotic was tested according to the following criteria: determination of appearance, microbiological purity (bacterioscopic control and absence of foreign microflora), harmlessness, and specific activity (number of live bacteria in one dose of the drug).

Microbiological purity was determined according to DSTU 4483:2005 (DSSU, 2005).

The number of live microbial cells in one dose of the symbiotic product was determined by the method of serial dilutions in saline followed by inoculation of 0.1 cm^3 of bacteria from dilutions of 10^6 on De Man–Rogosa–Sharpe agar (MRS agar) and Blaurock medium.

Cultivation of lactobacilli and bifidobacteria was carried out on MRS agar, Blaurock media, and skim milk for 24–48 h at a temperature of 37 °C.

The determination of biochemical activity was carried out by a generally accepted method.

The symbiotic product should be harmless to white mice weighing 20 ± 1 g when administered orally in an amount corresponding to one dose of the product. The symbiotic was dissolved with 0.9% sodium chloride solution at the rate of 0.5 cm^3 per dose. The resulting solution was orally administered to 15 mice weighing 20 ± 1 g in the stomach (using a special nozzle for a 1 cm^3 syringe) — 0.5 cm^3 each. The mice were observed for 21 days.

All experiments were performed in triplicate. The results were processed by methods of variation statistics using Microsoft Excel for Windows 2010. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results. For the production of a symbiotic biologically active supplement for animals, a formulation and technological regulations for its manufacture were developed. The formulations of the symbiotic are presented in Table 1.

Table 1 — The studied formulations of symbiotic biologically active supplement for animals

Components	Formulation number					
	1	2	3	4	5	6
Lactulose, %	1.0 ± 0.02	1.2 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Inulin, %	1.0 ± 0.02	1.0 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Fructose, %	1.0 ± 0.02	1.0 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Starch, %	57.0 ± 3.06	52.0 ± 3.01	45.0 ± 2.17	40.0 ± 3.06	36.5 ± 2.04	31.0 ± 2.01
Species and dosage of lactic acid bacteria	A mixture of dried cultures of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316, not less than 1×10^7 CFU/cm^3					

The ratio of symbiotic components was chosen based on our previous research.

The critical parameter of a symbiotic with a probiotic component is the viability of lactic acid bacteria cells, so the first stage of our research was to determine the number of viable cells in each sample on the day of its manufacture. Our studies were carried out by direct inoculation of the corresponding sample of the product onto the surface of the dense MRS agar. As a control, we used the inoculation of lactobacilli and bifidobacteria dissolved in saline at the same concentration as in the samples with 10^6 CFU/cm^3. The results of the experiments are shown in Table 2.

Table 2 — The number of viable cells of lactic acid bacteria in the experimental samples

The studied formulations of symbiotic biologically active supplement	The number of viable cells, CFU/cm^3
Formulation No. 1	$3.5 \pm 0.11 \times 10^7$
Formulation No. 2	$4.2 \pm 0.13 \times 10^8$
Formulation No. 3	$3.8 \pm 0.14 \times 10^9$
Formulation No. 4	$4.4 \pm 0.15 \times 10^7$
Formulation No. 5	$4.9 \pm 0.13 \times 10^8$
Formulation No. 6	$3.2 \pm 0.18 \times 10^6$
Control *Lactobacillus plantarum* No. 7-317	$3.7 \pm 0.12 \times 10^8$
Control *Bifidobacterium adolescentis* No. 17-316	$4.1 \pm 0.17 \times 10^9$

The results of the experiments showed that the lowest number of viable cells was in sample No. 6 and amounted to $3.2 \pm 0.18 \times 10^6$ CFU/cm^3, which is less than the minimum permissible value of the number of viable cells — 1×10^7 CFU/cm^3. Therefore, this sample was excluded from further studies. Sample No. 3 had the best results, the number of viable cells was $3.8 \pm 0.14 \times 10^9$ CFU/cm^3.

During the long-term storage of the drug under normal conditions at room temperature, control studies were conducted every three months for 24 months of storage. It should be noted that the proposed shelf life of the developed symbiotic is 24 months, which is generally accepted for medicines containing live bacteria.

The microbiological purity of the samples during 24 months of storage at room temperature (20 ± 2 °C) is shown in Table 3.

The study of microbiological purity (Table 3) of samples of symbiotic biologically active supplement for animals showed that the number of contaminating aerobic microorganisms after 6 months of storage was no more than 100 CFU/cm^3, and the number of contaminating yeast and mold fungi was no more than 10 CFU/cm^3. It was experimentally confirmed that microorganisms of the *Staphylococcus aureus* and *Pseudomonas aeruginosa* families were absent.

The analysis of the microbiological purity of the developed product showed that in terms of microbial contamination with foreign microflora, the product meets the requirements of the Technical Specifications of Ukraine only when stored for 3 months.

Table 3 — Symbiotic test results for microbiological purity during storage at room temperature (20 ± 2 °C).

Shelf life	Total amount, CFU/cm^3			
	Aerobic microorganisms	Yeast and mold fungi	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
On the day of manufacture	Absent			
3 months				
6 months	< 100	< 10	Absent	Absent
9 months				
12 months				
15 months				
18 months				
21 months				
24 months				

The quality indicators of the developed symbiotic product, permissible norms, and control methods are summarized in Table 4.

To study the stability of the drug, and determine the storage conditions and shelf life, several experimental samples of the symbiotic were made and stored in the laboratory. Compliance tests were conducted every three months. The results of the symbiotic biologically active supplement analysis are shown in Tables 5 and 6.

Determination of the quality indicators of symbiotic samples for 24 months at storage temperatures of 20 ± 2 °C and 4 ± 0.5 °C showed a non-compliance with one or more indicators. Thus, during the 24 months of the study, when stored at room temperature, the symbiotic samples did not meet the quality control methods for 'Microbiological purity of a drug', 'Quantification of bifidobacteria and lactobacilli', and 'Biochemical activity of a symbiotic biologically active supplement'.

Table 4 — Norms and methods of control of symbiotic biologically active supplement for animals

Indicators	Results obtained	Control methods
Description of a symbiotic biologically active supplement	Homogeneous powder of white or cream color	Visually
Microbiological purity of a drug	The presence of bacterial and fungal microflora is not allowed. The preparation should contain only bifidobacteria and lactic acid bacteria. Gram-stained smears should contain gram-positive bacilli characteristic of bifidobacteria and lactobacilli.	According to DSTU 4483:2005 (DSSU, 2005)
Quantification of bifidobacteria and lactobacilli	Total number of bifidobacteria cells at the end of the expiration date — < 1×10^8 CFU/ cm^3. Total number of lactobacilli cells at the end of the expiration date — < 1×10^8 CFU/ cm^3.	According to the Technical Specifications of Ukraine
Biochemical activity of a symbiotic biologically active supplement	Diluted symbiotic biologically active supplement coagulates skim milk in 18–24 h when incubated in a thermostat at a temperature of 37 ± 0.5 °C	
Harmlessness of a symbiotic biologically active supplement	A symbiotic biologically active supplement should be harmless when tested on white mice	

Table 5 — Results of the analysis of a symbiotic biologically active supplement during long-term storage at room temperature (20 ± 2 °C)

Indicators	On the day of production	3 months	6 months	9 months	12 months	15 months	18 months	21 months	24 months
Description of a symbiotic biologically active supplement	Homogeneous white powder								
Microbiological purity of a drug	Complies with the requirements		Does not meet the requirements						
Quantification of bifidobacteria and lactobacilli	3.8 ± 0.14 × 10^9	3.1 ± 0.11 × 10^8	3.8 ± 0.14 × 10^6	2.7 ± 0.12 × 10^6	1.8 ± 0.15 × 10^6	1.7± 0.12 × 10^6	1.5 ± 0.11 × 10^6	1.1 ± 0.13 × 10^6	1.8 ± 0.20 × 10^5
Biochemical activity of a symbiotic biologically active supplement	Complies with the requirements					Does not meet the requirements			
Harmlessness of a symbiotic biologically active supplement	Complies with the requirements								

Table 6 — Results of the analysis of a symbiotic biologically active supplement during long-term storage at a temperature of 4 ± 0.5 °C

Indicators	On the day of production	3 months	6 months	9 months	12 months	15 months	18 months	21 months	24 months
Description of a symbiotic biologically active supplement	Homogeneous white powder								
Microbiological purity of a drug	Complies with the requirements								
Quantification of bifidobacteria and lactobacilli	$3.8 \pm 0.14 \times 10^9$	$3.7 \pm 0.12 \times 10^9$	$3.5 \pm 0.10 \times 10^9$	$3.1 \pm 0.17 \times 10^9$	$3.9 \pm 0.14 \times 10^8$	$3.2 \pm 0.11 \times 10^8$	$2.9 \pm 0.12 \times 10^8$	$2.6 \pm 0.15 \times 10^8$	$1.7 \pm 0.13 \times 10^8$
Biochemical activity of a symbiotic biologically active supplement	Complies with the requirements								
Harmlessness of a symbiotic biologically active supplement	Complies with the requirements								

It should be noted that when stored in a refrigerator at a temperature of 4 ± 0.5 °C, the samples did not meet the test for 'Quantification of lactobacilli and bifidobacteria', but met the standard for 24 months. Thus, the symbiotic biologically active supplement for animals remains stable for 24 months at a storage temperature of 4 ± 0.5 °C and for 3 months at room temperature (20 ± 2 °C).

Thus, the recommended shelf life of the symbiotic biologically active supplement for animals is 24 months at a temperature of 4 ± 0.5 °C. The quality indicators of the product during 24 months of observation did not change compared to the results at the time of manufacture, which indicates a rationally based formulation of the product and its production technology. It should also be noted that the symbiotic can be stored at room temperature (20 ± 2 °C) for 3 months.

Conclusions. The results show that the symbiotic biologically active supplement for animals on the day of manufacture and after 3, 6, 9, 12, 15, 18, 21, and 24 months retained its biochemical activity and was harmless to white mice throughout the study period when stored at a temperature of 4 ± 0.5 °C. In addition, the symbiotic remains usable for 3 months after manufacture when stored at room temperature (20 ± 2 °C).

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ANTAGONISTIC ACTIVITY OF PROBIOTIC *BACILLUS* STRAINS ON PLANKTONIC FORMS OF BIOFILM-FORMING BACTERIA AND FUNGI

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Summary. The presence of microbial biofilms of pathogenic fungi and bacterial contaminants in animal feed can lead to disruption of the intestinal microflora and the development of infectious diseases. A promising field of study is the examination of the antagonistic effect of bacteria from the genus *Bacillus* on microbial biofilms and planktonic forms of pathogenic microorganisms in feed. The objective of this research is to investigate the antimicrobial and antifungal activity of the probiotic complex of bacteria from the genus *Bacillus* against planktonic forms of biofilm-forming pathogenic fungi and microorganisms isolated from pig feed. The antagonistic activity of five probiotic strains against the test cultures *Pasteurella multocida* type D No. 07, *Neisseria meningitidis* No. 18, *Streptococcus haemolyticus* No. 14, *Escherichia coli* No. 21, *Actinobacillus pleuropneumoniae* No. 12 was studied by the method of delayed inoculation (perpendicular strokes) in three replicates. The antifungal activity against the test fungi *Aspergillus niger* No. 1 and *Aspergillus candidus* No. 2 was evaluated by the injection method. According to the results of the study, it was determined that the strain *B. licheniformis* UNCSM-033 showed a pronounced antagonistic effect on the bacteria *N. meningitidis* No. 18 with an inhibition level of 26.7 ± 1.2 mm. Inhibition of growth and reproduction of *S. haemolyticus* No. 14 at a high level of intensity was determined in four probiotic strains with diameters of growth inhibition from 28.7 ± 1.2 mm to 34.0 ± 1.2 mm. A sufficiently high level of antagonism against the test culture *E. coli* No. 21 was shown by five experimental probiotic strains in the range of 24.7 ± 1.2 mm to 30.7 ± 2.3 mm, respectively. A very high level of antagonistic properties of the probiotic complex of bacteria of the genus *Bacillus* from five experimental strains against five types of pathogenic microorganisms from 38.3 ± 0.9 mm was shown in *A. pleuropneumoniae* No. 12 and up to 47.3 ± 0.9 mm in *P. multocida* type D No. 07. The highest degree of antagonistic activity against five test cultures of biofilm-forming microorganisms and antifungal effect against two test strains of pathogenic fungi, *A. niger* No. 1 and *A. candidus* No. 2, was demonstrated by the probiotic complex of bacteria belonging to the genus *Bacillus* (five strains). The pronounced antimicrobial properties of the five strains of the probiotic complex of bacteria of the genus *Bacillus* allow for the potential development of drugs based on them as an alternative to antibiotics

Keywords: antifungal effect, antibiotic substances, inhibitory activity

Introduction. The organization of animal feeding should provide conditions that facilitate the efficient use of feed and regulate microbiological digestive processes. Incomplete and unbalanced diets, as well as animal feeding with contaminated feed containing microbial biofilms of pathogenic fungi and bacterial contaminants, can disrupt the intestinal microflora and lead to the development of infectious diseases. This, in turn, can result in dysbiosis in pigs, which may reduce their natural resistance and productivity. One potential solution to this problem is the inclusion of probiotics in animal diets. Spore-forming bacteria from the genus *Bacillus*, which exhibit antagonistic activity, are increasingly used as probiotic cultures against planktonic forms and biofilms of pathogenic and opportunistic microorganisms of the genera *Escherichia*, *Salmonella*, *Shigella*, *Serratia*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Citrobacter*, *Candida* and *Aspergillus* fungi by producing antibiotics, bacteriocins, lysozyme, participate in digestion by synthesizing hydrolytic enzymes (analogues of macroorganism digestive enzymes) and at the same time do not inhibit the growth of lacto- and bifidobacteria (Kolchyk et al., 2022; Petrova and Sauer, 2016). Antagonism is ensured by the production of more than 200 antibiotic substances (polymyxins, bacitracins, gramicidin C, subtilin, microbacillin, biosporicin). Participation in the digestive process occurs due to the synthesis of extracellular hydrolytic enzymes proteases, amylases, pectinases, cellulases, and lipases. Probiotic functions are performed not only by vegetative bacilli cells but also by germinating spores (Cutting, 2011; Kotowicz et al., 2019; Chechet et al., 2022).

The vast majority of *Bacillus* species are harmless to animals. Strains that produce toxins and have pronounced adhesive and invasive properties are not used in the production of probiotics.

A promising area is the study of the antagonistic effect of bacteria from the genus *Bacillus* on microbial biofilms and planktonic forms of pathogenic microflora of feed for the further design of probiotic agents to break the epizootic chain of not only bacterial but also viral infections in pig production.

The study aimed to investigate the antimicrobial and antifungal activity of a probiotic complex of bacteria of the genus *Bacillus* against planktonic forms of biofilm-

forming pathogenic fungi and microorganisms isolated from pig feed.

Materials and methods. Microbiological studies of feeds were carried out in the Laboratory for Pig Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) using modern methods. The object of the study was the probiotic complex of bacteria of the genus *Bacillus*, namely five strains in its composition (*B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031 and *B. licheniformis* UNCSM-033).

The test cultures of pathogenic and opportunistic bacteria *Pasteurella multocida* type D No. 07, *Neisseria meningitidis* No. 18, *Streptococcus haemolyticus* No. 14, *Escherichia coli* No. 21, *Actinobacillus pleuropneumoniae* No. 12 were used as test cultures for determining the antagonistic activity of the five strains of the probiotic complex of bacteria of the genus *Bacillus in vitro.* To determine the antifungal activity, micromycetes of the genus *Aspergillus*, namely *A. niger* No. 1 and *A. candidus* No. 2, were used. Field isolates of the above microorganisms were isolated from 38 industrial batches of feed (grain, barley, and corn) from four pig farms positive for reproductive and neonatal infections in pigs in two regions of Ukraine.

Cultivation of five strains of the probiotic complex of bacteria of the genus *Bacillus* was carried out on meat-peptone broth (MPB) and meat-peptone agar (MPA) with the addition of 1% glucose, test cultures of microorganisms: *E. coli* No. 21 and *N. meningitidis* No. 18 — MPB, MPA, Endo agar; *P. multocida* type D No. 07 — Hottinger broth and agar; *S. haemolyticus* No. 14 — 5% blood agar; *A. pleuropneumoniae* No. 12 — 5% blood agar with 10% yeast extract; *A. niger* No. 1 and *A. candidus* No. 2 — Czapek medium.

The antagonistic activity of *B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, and *B. licheniformis* UNCSM-033 strains was studied by the method of delayed inoculation (perpendicular strokes) in three replicates (Ivchenko, 2004). To determine the antagonistic activity, the antagonist strains were inoculated on the surface of the agar and a day later, after incubation at a temperature of 37.0 ± 0.5 °C, the test culture of microorganisms was inoculated perpendicularly to it at a distance of no more than 2 mm. The growth of the test cultures was controlled by their parallel inoculation on plates with the same media but without antagonist strains.

The activity of bacilli antagonists to the test fungi *A. niger* No. 1 and *A. candidus* No. 2 was evaluated by the injection method (Sidorova, 2020). A suspension of fungal spores of each species of the test system was washed off with sterile water from 10-day-old colonies (100 μL at a spore density of 10^5 spores/ml) and spread over the surface of the Petri dish medium on Czapek agar. Then, 24-hours-old biomass of antagonist bacteria was inoculated onto the agar surface. The plates were incubated at a temperature of 28.0 ± 0.5 °C for three days.

The antifungal activity of five strains of the probiotic complex of bacteria belonging to the genus *Bacillus* was evaluated by measuring the diameter of the inhibition zone around the colonies of isolates of microorganisms and pathogenic fungi.

Results. The results of the study on antagonistic activity revealed that a high level of growth inhibition of the test culture *P. multocida* type D No. 07 was observed in all five strains of the probiotic complex of bacteria belonging to the genus *Bacillus*. This was evidenced by the diameter of the growth inhibition zones, which ranged from 27.3 ± 0.9 mm to 35.3 ± 2.0 mm (Table 1).

Table 1 — Antagonistic activity of five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* against planktonic forms of biofilm-forming microorganisms *in vitro*

Bacteria culture	Diameter of the zone of inhibition of growth of biofilm-forming microorganisms, mm				
	P. multocida type D No. 07	*N. meningitidis* No. 18	*S. haemolyticus* No. 14	*E. coli* No. 21	*A. pleuropneumoniae* No. 12
B. amyloliquefaciens ALB 65	27.3 ± 0.9	17.7 ± 1.2	28.7 ± 1.2	26.3 ± 1.5	16.3 ± 0.3
B. pumilus UNCSM-026	35.3 ± 2.0	22.0 ± 1.7	21.3 ± 2.3	29.3 ± 0.9	22.3 ± 0.9
B. subtilis UNCSM-020	29.3 ± 1.5	14.3 ± 3.2	32.3 ± 2.6	24.7 ± 1.2	18.7 ± 1.2
B. subtilis var. *mesentericus* UNCSM-031	34.0 ± 2.3	23.3 ± 2.6	28.7 ± 0.7	27.3 ± 2.0	26.0 ± 0.6
B. licheniformis UNCSM-033	31.3 ± 3.8	26.7 ± 1.2	34.0 ± 1.2	30.7 ± 2.3	20.3 ± 1.5
Probiotic complex of bacteria of the genus *Bacillus* (five strains)	47.3 ± 0.9	40.3 ± 2.6	45.3 ± 0.9	39.3 ± 1.5	38.3 ± 0.9

Note. Inhibition level, mm: up to 13 — low; 14–26 — medium; 27–36 — high; 37 and more — very high.

The *B. licheniformis* UNCSM-033 strain demonstrated a notable antagonistic impact on *N. meningitidis* No. 18, with an inhibition level of 26.7 ± 1.2 mm. This was higher than the corresponding value of the *B. subtilis* var. *mesentericus* UNCSM-031 strain by 12.7%, *B. pumilus* UNCSM-026 by 17.6%, *B. amyloliquefaciens* ALB 65 by 33.7% and *B. subtilis* UNCSM-020 by 46.4%, respectively.

Inhibition of growth and reproduction of *S. haemolyticus* No. 14 at a high level of intensity was determined in four probiotic strains *B. licheniformis* UNCSM-033 — 34.0 ± 1.2 mm, *B. subtilis* UNCSM-020 — 32.3 ± 2.6 mm, *B. amyloliquefaciens* ALB 65 — 28.7 ± 1.2 mm and *B. subtilis* var. *mesentericus* UNCSM-031 — 28.7 ± 0.7 mm, while the strain *B. pumilus* UNCSM-026 showed an average inhibition level of 21.3 ± 2.3 mm.

A sufficiently high level of antagonism concerning the test culture *E. coli* No. 21 was shown by five experimental probiotic strains. Thus, the spore-forming strain *B. licheniformis* UNCSM-033 with a diameter of growth inhibition 30.7 ± 2.3 mm did not have a significant difference from the values of *B. pumilus* UNCSM-026 (29.3 ± 0.9 mm), *B. subtilis* var. *mesentericus* UNCSM-031 (27.3 ± 2.0 mm), *B. amyloliquefaciens* ALB 65 (26.3 ± 1.5 mm) and *B. subtilis* UNCSM-020 (24.7 ± 1.2 mm), respectively.

Antimicrobial activity concerning *A. pleuropneumoniae* No. 12 was observed in all experimental probiotic strains with an average level of antagonistic effect: in strain *B. amyloliquefaciens* ALB 65 (16.3 ± 0.3 mm) and differed from this indicator in *B. subtilis* UNCSM-020 by 14.7%, in *B. licheniformis* UNCSM-033 by 24.5%, *B. pumilus* UNCSM-026 by 36.8% and *B. subtilis* var. *mesentericus* UNCSM-031 by 59.5%, respectively. In addition, it is necessary to note a very high level of antagonistic properties of the probiotic complex of bacteria of the genus *Bacillus* from five experimental strains against five types of pathogenic microorganisms from 38.3 ± 0.9 mm in *A. pleuropneumoniae* No. 12 and up to 47.3 ± 0.9 mm inhibition in *P. multocida* type D No. 07.

At the next research stage, the results of the antifungal activity of five probiotic strains of *Bacillus* were obtained. As a result of the studies, it was found that the average level of antifungal activity was observed in two probiotic strains *B. subtilis* var. *mesentericus* UNCSM-031 (15.7 ± 0.7 mm and 19.3 ± 2.6 mm) and *B. licheniformis* UNCSM-033 (17.3 ± 2.0 mm and 24.7 ± 0.7 mm) against pathogenic fungi *A. niger* No. 1 and *A. candidus* No. 2. Strains *B. amyloliquefaciens* ALB 65 (23.3 ± 0.9 mm) and *B. pumilus* UNCSM-026 (26.0 ± 1.7 mm) had an average level of inhibition only against *A. niger* No. 1, respectively (Table 2).

Table 2 — Antagonistic activity of five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* against pathogenic fungi *in vitro*

Bacteria culture	Diameter of the zone of inhibition of pathogenic fungi growth, mm	
	A. niger No. 1	*A. candidus* No. 2
B. amyloliquefaciens ALB 65	23.3 ± 0.9	31.0 ± 1.2
B. pumilus UNCSM-026	26.0 ± 1.7	32.3 ± 0.9
B. subtilis UNCSM-020	28.3 ± 0.9	33.3 ± 2.2
B. subtilis var. *mesentericus* UNCSM-031	15.7 ± 0.7	19.3 ± 2.6
B. licheniformis UNCSM-033	17.3 ± 2.0	24.7 ± 0.7
Probiotic complex of bacteria of the genus *Bacillus* (five strains)	30.7 ± 1.2	37.8 ± 2.0

Note. Inhibition level, mm: up to 13 — low; 14–26 — medium; 27–36 — high; 37 and more — very high.

High fungicidal activity was observed in the probiotic strain *B. subtilis* UNCSM-020 with a zone of growth inhibition of 28.3 ± 0.9 mm against *A. niger* No. 1, and against *A. candidus* No. 2 this value was higher by 17.7%. Two probiotic strains *B. amyloliquefaciens* ALB 65 (31.0 ± 1.2 mm) and *B. pumilus* UNCSM-026 (32.3 ± 0.9 mm) had a high level of mycelial destruction in *A. candidus* No. 2. The highest level of antifungal activity was found in the probiotic complex of bacteria of the genus *Bacillus* (37.8 ± 2.0 mm) against the fungal strain *A. candidus* No. 2 and high against *A. niger* No. 1 (30.7 ± 1.2 mm), respectively.

Thus, according to the results of the studies, five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* (*B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, and *B. licheniformis* UNCSM-033) demonstrated antagonistic activity against opportunistic and pathogenic microorganisms and fungicidal effect against pathogenic fungi. The most sensitive to the five antagonist strains were the test strains of *P. multocida* type D No. 07, *S. haemolyticus* No. 14 and *E. coli* No. 21. The highest degree of antagonistic activity against five test cultures of biofilm-forming microorganisms and antifungal effect against two test strains of pathogenic fungi *A. niger* No. 1 and *A. candidus* No. 2 was shown by the probiotic complex of bacteria of the genus *Bacillus* (five strains).

Discussion. The antimicrobial properties of *Bacillus* strains of the probiotic complex are due to their ability to destroy (lyse) certain bonds in the peptidoglycan structure of cell walls of different microorganism types. The mechanism of antagonistic action of probiotic strains is due to the presence of their properties useful for the macroorganism, in particular, the ability to synthesize various biologically active compounds (Cairns, Hobley and Stanley-Wall, 2014; Sumi et al., 2015; Khardziani et al., 2017). These include antibiotic substances and extracellular enzymes, including proteases, amino acids, and polysaccharides. *B. subtilis* and *B. licheniformis* strains can synthesize cyclic lipopeptide antibiotics. The main components of these antibiotics are surfactin, fengicin, and bacitracin, which are characterized by multiple biological activities (AlGburi et al., 2017; Chen et al., 2024). Lipopeptides from bacilli also exhibit pronounced inhibitory activity against various types of pathogenic fungi.

The antimicrobial activity of bacilli is determined not only by the synthesis of antibiotics, but also by lytic enzymes that cause the lysis of cells of gram-positive and gram-negative bacteria, as well as pathogenic fungi, which together or separately are capable of destroying the cell wall of microorganisms (Adeniji, Aremu and Babalola, 2019; Kadhum and Hasan, 2019; Mardonov et al., 2021)

In addition, bacilli can form biofilms and form associations with several microorganisms (five antagonist strains) and thereby inhibit the rate of biofilm formation in test cultures of *P. multocida* type D No. 07, *N. meningitidis* No. 18, *S. haemolyticus* No. 14, *E. coli* No. 21, *A. pleuropneumoniae* No. 12 (Aguilar et al., 2010; Arnaouteli et al., 2021). Taken together, the results confirm the probiotic potential of *Bacillus* bacteria, emphasizing its metabolic advantage over pathogens.

Conclusions. 1. The strains *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, *B. licheniformis* UNCSM-033 have versatile inhibitory and fungicidal activity against opportunistic and pathogenic test cultures of microorganisms. A very high antagonistic activity (38.3–47.3 mm) was found in the probiotic complex of bacteria of the genus *Bacillus* to planktonic forms of test cultures of *P. multocida* type D No. 07, *N. meningitidis* No. 18, *S. haemolyticus* No. 14, *E. coli* No. 21, *A. pleuropneumoniae* No. 12 and biofilm-forming fungi *A. candidus* No. 2 (37.8 mm).

2. The pronounced antimicrobial properties of five strains of the probiotic complex of bacteria of the *Bacillus* genus present a promising avenue for developing drugs based on them as an alternative to antibiotics. This is particularly crucial in the context of the growing prevalence of antibiotic-resistant pathogens and the declining efficacy of numerous antibiotics.

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Part 3. Biosafety

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INTERFERENCE BETWEEN MOSQUITO DENSONUCLEOSIS VIRUS AND CERTAIN ARBOVIRUSES

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Summary. The active ingredient of the preparation Viroden, developed in Ukraine, is the mosquito densovirus. This virus has a wide tissue tropism and affects all phases of ontogenesis. It reproduces itself in the mosquito's salivary gland cells, but unlike arboviruses, it is harmless for humans and vertebrates. It is well established that simultaneous infection of an insect with different viruses is often accompanied by the phenomenon of interference, whereby the reproduction of one or both viruses is suppressed in the insect's body. Consequently, it was reasonable to investigate the results of concurrent infection with an arbovirus and a mosquito densovirus. Laboratory experiments demonstrated that mosquito densovirus suppressed the reproduction of West Nile, Sindbis, and Batai viruses in the mosquito's body, resulting in a significant decrease in their infective titers as well as a reduction in the transmission factor during blood-feeding. The relevance of this research is determined by the increasing levels of biological threats posed by zoonotic transmissible viral infections common to humans and animals. According to the predictions of experts, in light of the processes of globalization and climate change, this may result in the emergence of new pandemics and panzootics

Keywords: mosquito densovirus, West Nile virus, Sindbis virus, Batai virus

Introduction. Densonucleosis viruses (DNVs) are invertebrate-specific viruses belonging to the subfamily Densovirinae within the family Parvoviridae. Densonucleosis is characterized by the hypertrophy of the nuclei in infected cells and cytopathology that leads to either death or loss of vital functions in all stages of infected organisms. Mosquito densoviruses are mosquito-specific, entomopathogenic icosahedral, nonenveloped viruses with a diameter of 20–25 nm. Their single-stranded linear DNA genome ranges from 4 to 6 kilobases and ends in two hairpin structures (Li et al., 2019). All mosquito densoviruses are classified in the genus *Brevidensovirus* (Cotmore et al., 2014).

The mosquito densovirus was first described in a laboratory culture of *Aedes aegypti* (L.) mosquitoes maintained at the Taras Shevchenko National University of Kyiv (Lebedeva et al., 1973). Subsequent studies of its genome revealed that this virus belongs to the Parvoviridae family (Buchatsky and Filenko, 1988; Galev et al., 1989). Subsequently, DNVs were identified in laboratory cultures of mosquitoes belonging to the genus *Aedes* in Thailand (Kittayapong, Baisley and O'Neill, 1999) and in nature in countries such as India (Sivaram et al., 2009), China (Li et al., 2019), and Brazil (Ferreira et al., 2020). Additionally, DNVs have been isolated from C6/36 mosquito cells obtained from *Ae. aegypti* mosquitoes (Jousset et al., 1993; Barreau, Jousset and Cornet, 1994; Boublik et al., 1994; Mossimann et al., 2011; Cataneo et al., 2019). Furthermore, densoviruses have been identified in other mosquito species, including *Culex pipiens* (Jousset, Baquerizo and Bergoin, 2000; Zhai et al., 2008), *Toxorhynchites splendens* (Pattanakitsakul et al., 2007), and *Anopheles minimus* (Rwegoshora, Baisley and Kittayapong, 2000). It is well established that mosquitoes are vectors for numerous pathogens that can cause disease in humans and animals. This has led to significant interest in Ukraine and internationally. Over many years, extensive research has led to the identification of 11 species of arbovirus present in Ukraine, responsible for 25% of seasonal fever diseases (Vynohrad et al., 1994). Among these are West Nile (WN), Batai (Bt), and Sindbis (Sb) viruses, which have a high prevalence and a severe course of disease. The disease may range from a flu-like state to meningitis and encephalitis, with a lethal outcome (Vynohrad I. et al., 1994, 1996). The natural foci of these diseases have been identified in numerous districts, particularly in the steppe zone, where WN, Bt, Sb viruses are prevalent; in the forest zone (woodlands), where Bt and Sb viruses are also found; and in the Transcarpathian Region, where WN and Sb viruses are present. These arboviruses have been identified in numerous species of mosquitoes belonging to the subfamily Culicinae, which are prevalent in Eastern Europe. *Aedes communis*, *Ae. punctor*, *Ae. vexans*, *Ae. cantans*, *Ae. excrucians*, *Ae. caspius*, *Culex pipiens*, and *Culiseta annulata* (Vynohrad et al., 1996) are among the species that are likely to participate in arbovirus circulation in the natural foci. This explains the

maintenance of the tight epidemiological situation in these territories.

The mosquito densovirus is capable of replicating within the mosquito, exhibiting a wide tissue tropism. It affects all phases of ontogenesis (Kuznetsova and Buchatsky, 1988; Buchatsky, 1989). Unlike arboviruses, which also replicate within the mosquito's salivary gland cells, the mosquito densovirus is harmless to humans and vertebrates (Lebedinets, Vasi'ieva and Buchatsky, 1976; Vasil'eva et al., 1990). It is well established that the simultaneous infection of an insect with different viruses is often accompanied by the phenomenon of interference, whereby the reproduction of one or both viruses is suppressed in the insect's body (Kelly, 1980). Consequently, it was reasonable to investigate the results of concurrent infection with an arbovirus and a mosquito densovirus.

The aim of the study was to investigate the nature of the interaction between arboviruses and an entomopathogenic densovirus and to determine the effect of the latter on the ability of the mosquito to transmit arboviruses. The overall aim was to evaluate the possibility of using densovirus as a biological agent for unspecific prophylaxis of arboviral infections.

Materials and methods. The following arboviruses were used in this study: West Nile virus (Flaviviridae, *Flavivirus*, antigenic complex of Japanese encephalitis virus), Batai virus (Peribunyaviridae, *Orthobunyavirus*, antigenic complex of Bunyamwera virus), and Sindbis virus (Togaviridae, *Alfavirus*, antigenic complex of Western equine encephalomyelitis virus), as well as densovirus of blood-sucking mosquitoes (Parvoviridae, *Brevidensovirus*). The mosquito *Aedes aegypti* from a laboratory population was used as a model vector. This species was chosen because of its high sensitivity to various arboviruses and densovirus.

The mosquitoes in the experimental group were infected with densovirus in later larval stages (III–IV) to maximize the number of adult insects that become infected with them. Healthy uninfected females served as controls. A total of 106 uninfected females and 104 female mosquitoes from the larvae previously infected with densovirus were used in the experiments. Females were infected with arboviruses 3–4 days after emergence by blood-feeding using a tampon wetted with 10% viral suspension in hemolyzed mouse blood. On the 10th, 15th, and 20th day after infection, the following parameters were determined:

(1) infective titer of arbovirus in mosquitoes;

(2) identification and localization of arbovirus in the salivary glands;

(3) the ability of the infected mosquitoes to transmit the arbovirus through a blood meal.

The quantity of arbovirus present in the suspension utilized for infection and in the infected mosquitoes was quantified by titration with a biological assay utilizing mice. For this method, one-to-two-day-old mice were inoculated in the brain with the viral suspension or mosquito tissue homogenate. The natural threshold dose for infecting the greatest number of mosquitoes with arboviruses is 0.6 to 3.5 log LD_{50}/0.03 ml. In the experimental conditions employed, the arboviral titers in the suspensions utilized for infecting mosquitoes were respectively 5.9–6.2 log LD_{50}/0.01 ml for WN virus; 5.4–6.0 log LD_{50}/0.01 ml for Sb virus; and 4.5–4.7 log LD_{50}/0.01 ml for Bt virus.

The localization of arboviral antigens in the salivary glands of female mosquitoes was determined using indirect immunofluorescence with glandular preparations. The ability of mosquitoes to transmit arboviruses through a blood meal was evaluated by individually feeding females on white mice. The transmission factor was determined by counting the mice exhibiting specific signs of arboviral infection. The presence of virus in their brains was confirmed by a biological assay.

The results were processed by methods of variation statistics using Statistica v. 9.0. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results. The amounts of the three arboviruses that had accumulated in densovirus-infected and control female mosquitoes were determined. The infective titers for all three viruses were significantly lower in the densovirus-infected mosquitoes (Table 1).

Table 1 — Influence of mosquito densovirus on arbovirus reproduction in mosquito's organism

Virus	Number of mosquitoes, specimens		Incubation period, days	Infectivity, log LD_{50}/0.01 ml	
	Experiment	Control		Experiment	Control
West Nile	10	10	10	5.0	6.2
	12	15	15	4.0	6.3
	12	15	20	4.1	6.3
Sindbis	10	8	10	4.0	4.8
	10	10	15	4.3	6.0
	10	10	20	4.2	5.8
Batai	12	8	10	3.6	4.2
	15	14	15	3.2	4.4
	15	14	20	3.05	4.6

Note. $P < 0.05$ in all experiments in relation to controls.

It should be noted that the maximal decrease in infectivity for WN and Sb viruses was observed on the 15th day, while for Bt virus, it was on the 20th day. Furthermore, the reduced infectivity of WN and Bt viruses was clearly observed for 10 days of the experiment, while in the controls, it remained almost

unchanged (for WN) or increased (for Bt). In contrast, Sb virus elicited a sharp elevation of the titer on the 15th day, which subsequently stabilized.

The transmission of the virus through blood-feeding occurs only when the virus overcomes the intestinal barrier and reproduces itself in mosquito tissues, including the salivary glands. The success of transmission depends on the accumulation of the virus in the apical cavities of the proximal areas in the glandular lateral lobes in amounts high enough for such transmission (the threshold dose).

To determine the localization of arboviruses in the salivary glands in control *Ae. aegypti* females, immunofluorescence was used. Specific fluorescence initially appeared in the proximal areas of the lateral lobes of the salivary glands in 52–69% of the individuals 10 days after infection with all three arboviruses. On the 15th day, the fluorescence became more intense and spread to all glandular cells in 90% of the mosquitoes in that group. By the 20th day, fluorescence was observed in every preparation.

The specific fluorescence observed in the salivary glands of females in the densovirus-infected group was observed in only some cases 10 days after infection. It spread to individual glandular cells and was less intense than that observed in the control group. By the 15th day, one-third of the females exhibited intense fluorescence in the proximal areas of the glandular lateral lobes, and some mosquitoes exhibited fluorescence throughout the salivary glands. The arboviral antigen was detected in only 8–10% of the females in the experimental group on the 20th day. The experiments with densovirus-infected females indicated a significantly reduced arboviral reproduction in the salivary glands. The accumulation of that virus in large concentrations occurred later and in a lower number of females in those experiments.

The transmission of arboviruses through a blood meal was also significantly lower in the experimental groups than in the control groups throughout the entire observation period (Table 2). The most notable difference was observed on the 10th day for WN virus, with a 6-fold reduction, on the 20th day for Sb virus, with a 10-fold reduction, and on the 10th day for Bt virus, with an 8-fold reduction. It is noteworthy that in the control group, an increase in the transmission factor was observed from the 10th day to the 20th day. In contrast, in the females infected with densovirus, the maximal ability for transmission was observed on the 15th day, followed by a subsequent decrease on the 20th day.

For instance, there has been a more than six-fold reduction in the transmission ability of Sb virus between the 15th and 20th day. A previous infection with densovirus had the least effect on arboviral transmission through a bite on the 15th day.

The infection with Sb virus led to the latest manifestation of the ability to lower transmission. It may be associated with slower reproduction of the virus and its lower accumulation in the female's salivary glands.

Table 2 — Influence of mosquito densovirus on arbovirus vector capability

Virus	Number of mosquitoes, specimens		Incubation period, days	Vector capability, %	
	Experiment	Control		Experiment	Control
West Nile	10	10	10	6.4	38.5
	15	15	15	38.2	85.7
	12	15	20	12.0	86.9
Sindbis	10	10	15	44.5	67.4
	10	10	20	7.5	75.6
Batai	12	8	10	2.0	16.6
	15	15	15	28.6	57.0

Note. $P < 0.05$ in all experiments in relation to controls.

Discussion. The results presented above provide compelling evidence for the interference between the entomopathogenic mosquito densovirus and arboviruses. In laboratory experiments, the suppression of reproduction of WN, Sb, and Bt viruses by densovirus results in a significant decrease in their infective titers and a reduction in the transmission factor during blood-feeding. Therefore, the interference of the densovirus with the studied viruses reduces the vector capacity of bloodsucking mosquitoes as transmitters of arboviral infections. The preparation Viroden, which was developed based on the densovirus (Buchatsky, et al., 1987), has demonstrated high efficacy in regulating the number of mosquitoes in both laboratory and field settings (Lebedinets et al., 1978; Kuznetsova and Buchatsky, 1988; Suchman et al., 2006; Carlson, Suchman and Buchatsky, 2006). The mosquito densovirus and the preparation Viroden based on it are completely safe for vertebrates (Lebedinets, Vasi'ieva and Buchatsky, 1976; Vasil'eva et al., 1990). The use of this drug can significantly reduce the vector potential of carriers of arboviral infections.

In light of the mounting challenges posed by climate change, the findings of this research offer promising avenues for addressing the pressing concerns of protecting people and animals from the real biological threats posed by Dengue, Zika, West Nile fever, Chikungunya viruses, and other transmissible mosquito-borne pathogens. As a consequence of rising temperatures, these diseases are spreading to new territories of the European Union (Jacob et al., 2018) and Ukraine (Ecodia, 2020; Vynograd and Shul, 2021). This represents a genuine threat of epidemics and epizootics of new, particularly dangerous diseases in these areas, which

necessitates the intensification of scientific research for the creation of new approaches to biological threat protection.

Conclusions. The suppression of reproduction of West Nile, Sindbis, and Batai viruses in the mosquito body by the mosquito densovirus results in a significant decrease in their infective titers, as well as a reduction in the transmission factor during blood-feeding in laboratory experiments.

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Contents

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Part 3. Biosafety